09/423035

24 Rec'd PCT/PTC 28 OCT 1999

ENZYMATIC DNA MOLECULES

Description

TECHNICAL FIELD

The present invention relates to nucleic acid enzymes or catalytic (enzymatic) DNA molecules that are capable of cleaving other nucleic acid molecules, particularly RNA. The present invention also relates to compositions containing the disclosed enzymatic DNA molecules and to methods of making and using such enzymes and compositions.

BACKGROUND

The need for catalysts that operate outside of their native context or which catalyze reactions that are not represented in nature has resulted in the development of "enzyme engineering" technology. The usual route taken in enzyme engineering has been a "rational design" approach, relying upon the understanding of natural enzymes to aid in the construction of new enzymes. Unfortunately, the state of proficiency in the areas of protein structure and chemistry is insufficient to make the generation of novel biological catalysts routine.

Recently, a different approach for developing novel catalysts has been applied. This method involves the construction of a heterogeneous pool of macromolecules and the application of an *in vitro* selection procedure to isolate molecules from the pool that catalyze the desired reaction. Selecting catalysts from a pool of macromolecules is not dependent on a comprehensive understanding of their structural and chemical properties. Accordingly, this process has been dubbed "irrational design" (Brenner et al., Proc.Natl.Acad.Sci. USA, 89:5381-5383, 1992).

        Most efforts to date involving the rational design of
enzymatic RNA molecules or ribozymes have not led to
molecules with fundamentally new or improved catalytic
function. However, the application of irrational design
5   methods via a process we have described as "directed
molecular evolution" or "*in vitro* evolution", which is
patterned after Darwinian evolution of organisms in nature,
has the potential to lead to the production of DNA molecules
that have desirable functional characteristics.

10   This technique has been applied with varying degrees of
success to RNA molecules in solution (see, e.g., Mills et
al, Proc.Natl.Acad.Sci. USA, 58:217, 1967; Green et al,
Nature, 347:406, 1990; Chowrira et al, Nature, 354:320,
1991; Joyce, Gene, 82:83, 1989; Beaudry et al, Science,
15   257:635-641, 1992; Robertson et al, Nature, 344:467, 1990),
as well as to RNAs bound to a ligand that is attached to a
solid support (Tuerk et al, Science, 249:505, 1990;
Ellington et al, Nature, 346:818, 1990). It has also been
applied to peptides attached directly to a solid support
20   (Lam et al, Nature, 354:82, 1991); and to peptide epitopes
expressed within a viral coat protein (Scott et al, Science,
249:386, 1990; Devlin et al, Science, 249:249, 1990; Cwirla
et al, Proc.Natl.Acad.Sci. USA, 87:6378, 1990).

        It has been more than a decade since the discovery of
25   catalytic RNA (Kruger et al, Cell, 31:147-157, 1982;
Guerrier-Takada et al, Cell, 35:849-857, 1983). The list of
known naturally-occurring ribozymes continues to grow (see
Cech, in The RNA World, Gesteland & Atkins (eds.), pp.
239-269, Cold Spring Harbor Laboratory Press, Cold Spring
30   Harbor, NY (1993); Pyle, Science, 261:709-714, 1993;
Symons, Curr.Opin.Struct.Biol., 4:322-330, 1994) and, in
recent years, has been augmented by synthetic ribozymes
obtained through *in vitro* evolution. (See Joyce,

Curr.Opin.Struct.Biol., 4:331-336, 1994; Breaker et al,
Trends Biotech., 12:268-275, 1994; Chapman et al, Curr.Opin.
Struct.Biol., 4:618-622, 1994).

It seems reasonable to assume that DNA can have
catalytic activity as well, considering that it contains
most of the same functional groups as RNA. However, with
the exception of certain viral genomes and replication
intermediates, nearly all of the DNA in biological organisms
occurs as a complete duplex, precluding it from adopting a
complex secondary and tertiary structure. Thus it is not
surprising that DNA enzymes have not been found in nature.

Until the advent of the present invention, the design,
synthesis and use of catalytic DNA molecules with
nucleotide-cleaving capabilities has not been disclosed or
demonstrated. Therefore, the discoveries and inventions
disclosed herein are particularly significant, in that they
highlight the potential of *in vitro* evolution as a means of
designing increasingly more efficient catalytic molecules,
including enzymatic DNA molecules that cleave other nucleic
acids, particularly RNA.

BRIEF SUMMARY OF THE INVENTION

The present invention describes a synthetic or
engineered (i.e., non-naturally-occurring) catalytic DNA
molecule (or enzymatic DNA molecule) capable of cleaving a
substrate nucleic acid (NA) sequence at a defined cleavage
site. The invention also contemplates an enzymatic DNA
molecule having an endonuclease activity.

A preferred catalytic DNA molecule has site-specific
endonuclease activity specific for a nucleotide sequence
defining a cleavage site in a preselected substrate nucleic
acid sequence. The DNA molecule has first and second

substrate binding regions flanking a core region, wherein
the first substrate binding region has a sequence
complementary to a first portion of the preselected
substrate nucleic acid sequence, and the second substrate
binding region has a sequence complementary to a second
portion of the preselected substrate nucleic acid sequence.
The core region has a sequence according to the formula:

    (I.)   T(stem)'AGC(stem)"Z,

wherein the (stem)' and (stem)" are each three
sequential nucleotides which when hybridized as a
(stem)':(stem)" pair comprise three base pairs including at
least two G:C pairs and wherein Z = WCGR or WCGAA, and W = A
or T and R = A or G. In a preferred embodiment, formula I
defines SEQ ID NO 120 (8-17).

Also contemplated is a core region having a sequence
according to the formula:

    RGGCTAGCHACAACGA (SEQ ID NO: 122)

    (II.)   ~~RGGGTAGGXACAACGA (SEQ ID NO)~~,

wherein X = T, C or A, and R = A or G. In a preferred
embodiment, formula I defines SEQ ID NO 121 (10-23).

In another embodiment, the endonuclease activity is
specific for a nucleotide sequence defining a cleavage site
comprising single-stranded nucleic acid in a substrate
nucleic acid sequence. In another preferred variation, the
cleavage site is double-stranded nucleic acid. Similarly,
substrate nucleic acid sequences may be single-stranded,
double-stranded, partially single- or double-stranded,
looped, or any combination thereof.

In another contemplated embodiment, the substrate
nucleic acid sequence includes one or more nucleotide
analogues. In one variation, the substrate nucleic acid
sequence is a portion of, or attached to, a larger molecule.

In various embodiments, the larger molecule is selected
from the group consisting of RNA, modified RNA, DNA,

modified DNA, nucleotide analogs, or composites thereof. In
another example, the larger molecule comprises a composite
of a nucleic acid sequence and a non-nucleic acid sequence.

In another embodiment, the invention contemplates that
a substrate nucleic acid sequence includes one or more
nucleotide analogs. A further variation contemplates that
the single stranded nucleic acid comprises RNA, DNA,
modified RNA, modified DNA, one or more nucleotide analogs,
or any composite thereof. In one embodiment of the
disclosed invention, the endonuclease activity comprises
hydrolytic cleavage of a phosphoester bond at the cleavage
site.

In various preferred embodiments, the catalytic DNA
molecules of the present invention are single-stranded in
whole or in part. These catalytic DNA molecules may
preferably assume a variety of shapes consistent with their
catalytic activity. Thus, in one variation, a catalytic DNA
molecule of the present invention includes one or more
hairpin loop structures. In yet another variation, a
catalytic DNA molecule may assume a shape similar to that of
"hammerhead" ribozymes. In still other embodiments, a
catalytic DNA molecule may assume a conformation similar to
that of *Tetrahymena thermophila* ribozymes, e.g., those
derived from group I introns.

Similarly, preferred catalytic DNA molecules of the
present invention are able to demonstrate site-specific
endonuclease activity irrespective of the original
orientation of the substrate molecule. Thus, in one
preferred embodiment, an enzymatic DNA molecule of the
present invention is able to cleave a substrate nucleic acid
sequence that is separate from the enzymatic DNA molecule --
i.e., it is not linked to the DNAzyme. In another preferred
embodiment, an enzymatic DNA molecule is able to cleave an

attached substrate nucleic acid sequence -- i.e., it is able to perform a reaction similar to self-cleavage.

The invention also contemplates enzymatic DNA molecules (catalytic DNA molecules, deoxyribozymes or DNAzymes) having endonuclease activity, whereby the endonuclease activity requires the presence of a divalent cation. In various preferred, alternative embodiments, the divalent cation is selected from the group consisting of Pb^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, and Ca^{2+}. Another variation contemplates that the endonuclease activity requires the presence of a monovalent cation. In such alternative embodiments, the monovalent cation is preferably selected from the group consisting of Na^+ and K^+.

In various preferred embodiments of the invention, an enzymatic DNA molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 3, SEQ ID NO 14; SEQ ID NO 15; SEQ ID NO 16; SEQ ID NO 17; SEQ ID NO 18; SEQ ID NO 19; SEQ ID NO 20; SEQ ID NO 21; and SEQ ID NO 22. In other preferred embodiments, a catalytic DNA molecule of the present invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 23; SEQ ID NO 24; SEQ ID NO 25; SEQ ID NO 26; SEQ ID NO 27; SEQ ID NO 28; SEQ ID NO 29; SEQ ID NO 30; SEQ ID NO 31; SEQ ID NO 32; SEQ ID NO 33; SEQ ID NO 34; SEQ ID NO 35; SEQ ID NO 36; SEQ ID NO 37; SEQ ID NO 38; and SEQ ID NO 39.

Another preferred embodiment contemplates that a catalytic DNA molecule of the present invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 50 and SEQ ID NO 51. In yet another preferred embodiment, a catalytic DNA molecule of the present invention comprises a nucleotide sequence selected from the group consisting of SEQ ID NOS 52 through 101. As disclosed herein, catalytic DNA molecules having sequences

substantially similar to those disclosed herein are also
contemplated. Thus, a wide variety of substitutions,
deletions, insertions, duplications and other mutations may
be made to the within-described molecules in order to

5   generate a variety of other useful enzymatic DNA molecules;
as long as said molecules display site-specific cleavage
activity as disclosed herein, they are within the boundaries
of this disclosure.

In a further variation of the present invention, an

10   enzymatic DNA molecule of the present invention preferably
has a substrate binding affinity of about 1 µM or less. In
another embodiment, an enzymatic DNA molecule of the present
invention binds substrate with a K_D of less than about 0.1
µM.

15   The present invention also discloses enzymatic DNA
molecules having useful turnover rates. In one embodiment,
the turnover rate is less than 5 hr^{-1}; in a preferred
embodiment, the rate is less than about 2 hr^{-1}; in a more
preferred embodiment, the rate is less than about 1hr^{-1}; in

20   an even more preferred embodiment, the turnover rate is
about 0.6 hr^{-1} or less.

In still another embodiment, an enzymatic DNA molecule
of the present invention displays a useful turnover rate
wherein the k_{obs} is less than 1 min^{-1}, preferably less than

25   0.1 min^{-1}; more preferably, less than 0.01 min^{-1}; and even
more preferably, less than 0.005 min^{-1}. In one variation,
the value of k_{obs} is approximately 0.002 min^{-1} or less.

The present invention also contemplates embodiments in
which the catalytic rate of the disclosed DNA enzymes is

30   fully optimized. Thus, in various preferred embodiments,
the K_m for reactions enhanced by the presence of Mg^{2+} is
approximately 0.5-20 mM, preferably about 1-10 mM, and more
preferably about 2-5 mM.

        The present invention also contemplates an embodiment
whereby the nucleotide sequence defining the cleavage site
comprises at least one nucleotide. In various other
preferred embodiments, a catalytic DNA molecule of the
5   present invention is able to recognize and cleave a
nucleotide sequence defining a cleavage site of two or more
nucleotides.

        In various preferred embodiments, an enzymatic DNA
molecule of the present invention comprises a conserved core
10   flanked by one or more substrate binding regions. In one
embodiment, an enzymatic DNA molecule includes first and
second substrate binding regions. In another embodiment, an
enzymatic DNA molecule includes two or more substrate
binding regions.

15   As noted previously, preferred catalytic DNA molecules
of the present invention may also include a conserved core.
In one preferred embodiment, the conserved core comprises
one or more conserved regions. In other preferred
variations, the one or more conserved regions include a
20   nucleotide sequence selected from the group consisting of
CG; CGA; AGCG; AGCCG; CAGCGAT; CTTGTTT; and CTTATTT (see,
e.g., Figure 3).

        In one embodiment of the invention, an enzymatic DNA
molecule of the present invention further comprises one or
25   more variable or spacer nucleotides between the conserved
regions in the conserved core. In another embodiment, an
enzymatic DNA molecule of the present invention further
comprises one or more variable or spacer nucleotides between
the conserved core and the substrate binding region.

30   In one variation, the first substrate binding region
preferably includes a nucleotide sequence selected from the
group consisting of CATCTCT; GCTCT; TTGCTTTTT; TGTCTTCTC;
TTGCTGCT; GCCATGCTTT (SEQ ID NO 40); CTCTATTTCT (SEQ ID NO

41); GTCGGCA; CATCTCTTC; and ACTTCT. In another preferred
variation, the second substrate binding region includes a
nucleotide sequence selected from the group consisting of
TATGTGACGCTA (SEQ ID NO 42); TATAGTCGTA (SEQ ID NO 43);
ATAGCGTATTA (SEQ ID NO 44); ATAGTTACGTCAT (SEQ ID NO 45);
AATAGTGAAGTGTT (SEQ ID NO 46); TATAGTGTA; ATAGTCGGT;
ATAGGCCCGGT (SEQ ID NO 47); AATAGTGAGGCTTG (SEQ ID NO 48);
and ATGNTG.

In various embodiments of the present invention, the
substrate binding regions vary in length. Thus, for
example, a substrate binding region may comprise a single
nucleotide to dozens of nucleotides. However, it is
understood that substrate binding regions of about 3-25
nucleotides in length, preferably about 3-15 nucleotides in
length, and more preferably about 3-10 nucleotides in length
are particularly preferred. In various embodiments, the
individual nucleotides in the substrate binding regions are
able to form complementary base pairs with the nucleotides
of the substrate molecules; in other embodiments,
noncomplementary base pairs are formed. A mixture of
complementary and noncomplementary base pairing is also
contemplated as falling within the scope of the disclosed
embodiments of the invention.

In another preferred embodiment, a catalytic DNA
molecule of the present invention may further comprise a
third substrate binding region. In some preferred
embodiments, the third region includes a nucleotide sequence
selected from the group consisting of TGTT; TGTTA; and
TGTTAG. Another preferred embodiment of the present
invention discloses an enzymatic DNA molecule further
comprising one or more variable or "spacer" regions between
the substrate binding regions.

In another disclosed embodiment, the present invention

contemplates a purified, synthetic enzymatic DNA molecule separated from other DNA molecules and oligonucleotides, the enzymatic DNA molecule having an endonuclease activity, wherein the endonuclease activity is specific for a

5   nucleotide sequence defining a cleavage site comprising single- or double-stranded nucleic acid in a substrate nucleic acid sequence. In one variation, a synthetic (or engineered) enzymatic DNA molecule having an endonuclease activity is disclosed, wherein the endonuclease activity is

10   specific for a nucleotide sequence defining a cleavage site consisting essentially of a single- or double-stranded region of a substrate nucleic acid sequence.

       In yet another embodiment, the invention contemplates an enzymatic DNA molecule comprising a deoxyribonucleotide

15   polymer having a catalytic activity for hydrolyzing a nucleic acid-containing substrate to produce substrate cleavage products. In one variation, the hydrolysis takes place in a site-specific manner. As noted previously, the polymer may be single-stranded, double-stranded, or some

20   combination of both.

       The invention further contemplates that the substrate comprises a nucleic acid sequence. In various embodiments, the nucleic acid sequence substrate comprises RNA, modified RNA, DNA, modified DNA, one or more nucleotide analogs, or

25   composites of any of the foregoing. One embodiment contemplates that the substrate includes a single-stranded segment; still another embodiment contemplates that the substrate is double-stranded.

       The present invention also contemplates an enzymatic

30   DNA molecule comprising a deoxyribonucleotide polymer having a catalytic activity for hydrolyzing a nucleic acid-containing substrate to produce a cleavage product. In one variation, the enzymatic DNA molecule has an effective

binding affinity for the substrate and lacks an effective
binding affinity for the cleavage product.

In one preferred embodiment, the invention discloses a
non-naturally-occurring enzymatic DNA molecule comprising a
5   nucleotide sequence defining a conserved core flanked by
recognition domains, variable regions, and spacer regions.
Thus, in one preferred embodiment, the nucleotide sequence
defines a first variable region contiguous or adjacent to
the 5'-terminus of the molecule, a first recognition domain
10   located 3'-terminal to the first variable region, a first
spacer region located 3'-terminal to the first recognition
domain, a first conserved region located 3'-terminal to the
first spacer region, a second spacer region located 3'-
terminal to the first conserved region, a second conserved
15   region located 3'-terminal to the second spacer region, a
second recognition domain located 3'-terminal to the second
conserved region, and a second variable region located 3'-
terminal to the second recognition domain.

In another embodiment, the nucleotide sequence
20   preferably defines a first variable region contiguous or
adjacent to the 5'-terminus of the molecule, a first
recognition domain located 3'-terminal to the first variable
region, a first spacer region located 3'-terminal to the
first recognition domain, a first conserved region located
25   3'-terminal to the first spacer region, a second spacer
region located 3'-terminal to the first conserved region, a
second conserved region located 3'-terminal to the second
spacer region, a second recognition domain located 3'-
terminal to the second conserved region, a second variable
30   region located 3'-terminal to the second recognition domain,
and a third recognition domain located 3'-terminal to the
second variable region.

In one variation of the foregoing, the molecule

includes a conserved core region flanked by two substrate
binding domains; in another, the conserved core region
comprises one or more conserved domains. In other preferred
embodiments, the conserved core region further comprises one
5   or more variable or spacer nucleotides. In yet another
embodiment, an enzymatic DNA molecule of the present
invention further comprises one or more spacer regions.

The present invention further contemplates a wide
variety of compositions. For example, compositions
10   including an enzymatic DNA molecule as described hereinabove
are disclosed and contemplated herein. In one alternative
embodiment, a composition according to the present invention
comprises two or more populations of enzymatic DNA molecules
as described above, wherein each population of enzymatic DNA
15   molecules is capable of cleaving a different sequence in a
substrate. In another variation, a composition comprises
two or more populations of enzymatic DNA molecules as
described hereinabove, wherein each population of enzymatic
DNA molecules is capable of recognizing a different
20   substrate. In various embodiments, it is also preferred
that compositions include a monovalent or divalent cation.

The present invention further contemplates methods of
generating, selecting, and isolating enzymatic DNA molecules
of the present invention. In one variation, a method of
25   selecting enzymatic DNA molecules that cleave a nucleic acid
sequence (e.g., RNA) at a specific site comprises the
following steps: (a) obtaining a population of putative
enzymatic DNA molecules -- whether the sequences are
naturally-occurring or synthetic -- and preferably, they are
30   single-stranded DNA molecules; (b) admixing nucleotide-
containing substrate sequences with the aforementioned
population of DNA molecules to form an admixture; (c)
maintaining the admixture for a sufficient period of time

and under predetermined reaction conditions to allow the putative enzymatic DNA molecules in the population to cause cleavage of the substrate sequences, thereby producing substrate cleavage products; (d) separating the population

5   of DNA molecules from the substrate sequences and substrate cleavage products; and (e) isolating DNA molecules that cleave substrate nucleic acid sequences (e.g., RNA) at a specific site from the population.

In a further variation of the foregoing method, the DNA

10   molecules that cleave substrate nucleic acid sequences at a specific site are tagged with an immobilizing agent. In one example, the agent comprises biotin.

In yet another variation of the aforementioned method, one begins by selecting a sequence -- e.g., a predetermined

15   "target" nucleotide sequence -- that one wishes to cleave using an enzymatic DNA molecule engineered for that purpose. Thus, in one embodiment, the pre-selected (or predetermined) "target" sequence is used to generate a population of DNA molecules capable of cleaving substrate nucleic acid

20   sequences at a specific site via attaching or "tagging" it to a deoxyribonucleic acid sequence containing one or more randomized sequences or segments. In one variation, the randomized sequence is about 40 nucleotides in length; in another variation, the randomized sequence is about 50

25   nucleotides in length. Randomized sequences that are 1-40, 40-50, and 50-100 nucleotides in length are also contemplated by the present invention.

In one embodiment of the present invention, the nucleotide sequence used to generate a population of

30   enzymatic DNA molecules is selected from the group consisting of SEQ ID NO 4, 23, 50 AND 51. In another embodiment, the "target" or "substrate" nucleotide sequence comprises a sequence of one or more ribonucleotides -- see,

-14-

e.g., the relevant portions of SEQ ID NOS 4 and 23, and SEQ
ID NO 49. It is also contemplated by the present invention
that a useful "target" or "substrate" nucleotide sequence
may comprise DNA, RNA, or a composite thereof.

5   The invention also contemplates methods as described
above, wherein the isolating step further comprises exposing
the tagged DNA molecules to a solid surface having avidin
linked thereto, whereby the tagged DNA molecules become
attached to the solid surface. As before, the substrate may
10   be RNA, DNA, a composite of both, or a molecule including
nucleotide sequences.

       The present invention also contemplates a method for
specifically cleaving a substrate nucleic acid sequence at a
particular cleavage site, comprising the steps of (a)
15   providing an enzymatic DNA molecule capable of cleaving a
substrate nucleic acid sequence at a specific cleavage site;
and (b) contacting the enzymatic DNA molecule with the
substrate nucleic acid sequence to cause specific cleavage
of the nucleic acid sequence at the cleavage site. In one
20   variation, the enzymatic DNA molecule is a non-naturally-
occurring (or synthetic) DNA molecule. In another
variation, the enzymatic DNA molecule is single-stranded.

       In still another variation of the foregoing method, the
substrate comprises a nucleic acid. In various embodiments,
25   the substrate nucleic acid comprises RNA, modified RNA, DNA,
modified DNA, one or more nucleotide analogs, or composites
of any of the foregoing. In yet another embodiment, the
specific cleavage is caused by the endonuclease activity of
the enzymatic DNA molecule. Alteration of reaction
30   conditions -- e.g., the adjustment of pH, temperature,
percent cation, percent enzyme, percent substrate, and
percent product -- is also contemplated herein.

       The present invention also contemplates a method of

cleaving a phosphoester bond, comprising (a) admixing an
catalytic DNA molecule capable of cleaving a substrate
nucleic acid sequence at a defined cleavage site with a
phosphoester bond-containing substrate, to form a reaction
5   admixture; and (b) maintaining the admixture under
predetermined reaction conditions to allow the enzymatic DNA
molecule to cleave the phosphoester bond, thereby producing
a population of substrate products. In one embodiment, the
enzymatic DNA molecule is able to cleave the phosphoester
10   bond in a site-specific manner. In another embodiment, the
method further comprises the steps of (c) separating the
products from the catalytic DNA molecule; and (d) adding
additional substrate to the enzymatic DNA molecule to form a
new reaction admixture.

15   The present invention also contemplates methods of
engineering enzymatic DNA molecules that cleave phosphoester
bonds. One exemplary method comprises the following steps:
(a) obtaining a population of single-stranded DNA molecules;
(b) introducing genetic variation into the population to
20   produce a variant population; (c) selecting individuals from
the variant population that meet predetermined selection
criteria; (d) separating the selected individuals from the
remainder of the variant population; and (e) amplifying the
selected individuals.

25

BRIEF DESCRIPTION OF THE DRAWINGS

       Figure 1 illustrates a selective amplification scheme
for isolation of DNAs that cleave a target RNA phosphoester.
As shown, double-stranded DNA that contains a stretch of 50
30   random nucleotides (the molecule with "N_{50}" indicated above
it) is amplified by PCR, employing a 5'-biotinylated DNA
primer that is terminated at the 3' end by an adenosine
ribonucleotide (rA). (The biotin label is indicated via the

encircled letter "B".) This primer is extended by *Taq*
polymerase to yield a DNA product that contains a single
embedded ribonucleotide. The resulting double-stranded DNA
is immobilized on a streptavidin matrix and the

5   unbiotinylated DNA strand is removed by washing with 0.2 N
NaOH. After re-equilibrating the column with a buffered
solution, the column is washed with the same solution with
added 1 mM PbOAc. DNAs that undergo Pb^{2+}-dependent
self-cleavage are released from the column, collected in the

10   eluant, and amplified by PCR. The PCR products are then
used to initiate the next round of selective amplification.

Figure 2 illustrates self-cleavage activity of the
starting pool of DNA (G0) and populations obtained after the
first through fifth rounds of selection (G1 - G5), in the

15   presence of lead cation (Pb^{2+}). The symbol **Pre** represents
108-nucleotide precursor DNA (SEQ ID NO 4); **Clv,**
28-nucleotide 5´-cleavage product (SEQ ID NO 5); and **M,**
primer 3a (SEQ ID NO 6), which corresponds in length to the
5´-cleavage product.

20   Figure 3 illustrates the sequence alignment of
individual variants isolated from the population after five
rounds of selection. The fixed substrate domain is shown at
(SEQ ID NOs 13-22)
the top, with the target riboadenylate identified via an
inverted triangle. Substrate nucleotides that are commonly

25   involved in presumed base-pairing interactions are indicated
by vertical bars. Sequences corresponding to the 50
initially-randomized nucleotides are aligned antiparallel to
the substrate domain. All of the variants are 3´-terminated
by the fixed sequence 5´-CGGTAAGCTTGGCAC-3´ (not shown; SEQ

30   ID NO 1). Nucleotides within the initially-randomized
region that are presumed to form base pairs with the
substrate domain are indicated on the right and left sides
of the Figure; the putative base-pair-forming regions of the

enzymatic DNA molecules are individually boxed in each
sequence shown. Conserved regions are illustrated via the
two large, centrally-located boxes.

     Figures 4A and 4B illustrate DNA-catalyzed cleavage of
5   an RNA phosphoester in an intermolecular reaction that
proceeds with catalytic turnover. Figure 4A is a
diagrammatic representation of the complex formed between
the 19mer substrate (3'-TCACTATrAGGAAGAGATGG-5', SEQ ID NO
2) and 38mer DNA enzyme (5'-
10   ACACATCTCTGAAGTAGCGCCGCCGTATAGTGACGCTA-3', SEQ ID NO 3).
The substrate contains a single adenosine ribonucleotide
("rA", adjacent to the arrow), flanked by
deoxyribonucleotides. The synthetic DNA enzyme is a
38-nucleotide portion of the most frequently occurring
15   variant shown in Figure 3. Highly-conserved nucleotides
located within the putative catalytic domain are "boxed".
As illustrated, one conserved sequence is "AGCG", while
another is "CG" (reading in the 5'→3' direction).

     Figure 4B shows an Eadie-Hofstee plot used to determine
20   K_m (negative slope) and V_{max} (y-intercept) for DNA-catalyzed
cleavage of [5'-^{32}P]-labeled substrate under conditions
identical to those employed during *in vitro* selection.
Initial rates of cleavage were determined for reactions
involving 5 nM DNA enzyme and either 0.125, 0.5, 1, 2, or 4
25   μM substrate.

     Figure 5 is a photographic representation showing a
polyacrylamide gel demonstrating specific endoribonuclease
activity of four families of selected catalytic DNAs.
Selection of a Pb^{2+}-dependent family of molecules was
30   repeated in a side-by-side fashion as a control (first
group). In the second group, Zn^{2+} is used as the cation; in
group three, the cation is Mn^{2+}; and in the fourth group,
the cation is Mg^{2+}. A fifth site on the gel consists of the

cleavage product alone, as a marker.

As noted, there are three lanes within each of the aforementioned four groups. In each group of three lanes, the first lane shows the lack of activity of the selected

5   population in the absence of the metal cation, the second lane shows the observed activity in the presence of the metal cation, and the third lane shows the lack of activity of the starting pool (G0).

Figures 6A and 6B provide two-dimensional illustrations

10 of a "progenitor" catalytic DNA molecule and one of several catalytic DNA molecules obtained via the selective amplification methods disclosed herein, respectively. Figure 6A illustrates an exemplary molecule from the starting pool, showing the overall configuration of the _from residue nos. 21-92_

15 molecules represented by SEQ ID NO 23. As illustrated, various complementary nucleotides flank the random (N_{40}) _(SEQ ID NO 123)_ region. Figure 6B is a diagrammatic representation of one of the Mg^{2+}-dependent catalytic DNA molecules (or "DNAzymes") generated via the within-described procedures.

20 The location of the ribonucleotide in the substrate nucleic acid is indicated via the arrow in both Figs. 6A and 6B.

Figure 7 illustrates some of the results of ten rounds of _in vitro_ selective amplification carried out essentially as described in Example 5 hereinbelow. As shown, two sites

25 and two families of catalysts emerged as displaying the most efficient cleavage of the target sequence. Cleavage conditions were essentially as indicated in Figure 7, namely, 10mM Mg^{2+}, pH 7.5, and 37°C; data collected after the reaction ran for 2 hours is shown. Cleavage (%) is

30 shown plotted against the number of generations (here, 0 through 10). The number/prevalence of catalytic DNA molecules capable of cleaving the target sequence at the indicated sites in the substrate is illustrated via the

(SEQ ID NO 49)

vertical bars, with cleavage at G|UAACUAGAGAU shown by the
striped bars, and with cleavage at GUAACUA|GAGAU illustrated (SEQ ID NO 49)
via the open (lightly-shaded) bars.

Figure 8 illustrates the nucleotide sequences, cleavage

5   sites, and turnover rates of two catalytic DNA molecules of
the present invention, clones 8-17 and 10-23. Reaction
conditions were as shown, namely, 10mM Mg^{2+}, pH 7.5, and
37°C. The DNAzyme identified as clone 8-17 $^{A'}$ insert is illustrated
on the left, with the site of cleavage of the RNA substrate

10   indicated by the arrow. The substrate sequence (5' -
GGAAAAAGUAACUAGAGAUGGAAG - 3') ^{A2} insert -- which is separate from the
DNAzyme (i.e., intermolecular cleavage is shown) -- is
labeled as such. Similarly, the DNAzyme identified herein
as 10-23 ^{A3} insert is shown on the right, with the site of cleavage of

15   the RNA substrate indicated by the arrow. Again, the
substrate sequence is indicated. For the 8-17 ^{A4} insert enzyme, the
turnover rate was approximately 0.6 hr^{-1}; for the 10-23
enzyme, the turnover rate was approximately 1 hr^{-1}.
Noncomplementary pairings are indicated with a closed circle

20   (●), whereas complementary pairings are indicated with a
vertical line (|).

Figure 9 further illustrates the nucleotide sequences,
cleavage sites, and turnover rates of two catalytic DNA
molecules of the present invention, clones 8-17 and 10-23.

25   Reaction conditions were as shown, namely, 10mM Mg^{2+}, pH
7.5, and 37°C. As in Figure 8, the DNAzyme identified as
clone 8-17 ^{A5} insert is illustrated on the left, with the site of
cleavage of the RNA substrate indicated by the arrow. The
substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3') ^{A6} insert --

30   which is separate from the DNAzyme (i.e., intermolecular
cleavage is shown) -- is labeled as such. Similarly, the
DNAzyme identified herein as 10-23 ^{A7} insert is shown on the right,
with the site of cleavage of the RNA substrate indicated by

-20-

the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme, k_{obs} was approximately 0.002 min^{-1}; for the 10-23 enzyme, the value of k_{obs} was approximately 0.01 min^{-1}. Noncomplementary pairings are indicated with a closed circle

5 (●), whereas complementary pairings are indicated with a vertical line (|).

Figure 10 illustrates a schematic showing the composition of the 8-17 and 10-23 catalytic motifs. The DNA (SEQ ID NO 122)

enzyme (bottom strand) binds the RNA substrate (top strand)

10 through complementary Watson-Crick pairing (vertical lines) between unspecified complementary nucleotides (horizontal lines). Cleavage occurs at the position indicated by the arrow, where R = A or G and Y = U or C.

Figure 11 illustrates the catalytic activity of the

15 10-23 DNA enzyme under multiple-turnover conditions as described in Example 6. Initial velocities were measured over the first 10% of the reaction, employing a fixed concentration of enzyme (0.004 nM) and varying concentrations of substrate (0.02-4 nM). The 17mer RNA

20 substrate, corresponding to the start codon region of HIV-1 gag / pol mRNA, was prepared by in vitro transcription. Reaction conditions: 2 mM MgCl2, 150 mM NaCl, pH 7.5, 37 C. Data from two independent experiments are shown and were fit to the Michaelis-Menten equation: v = kcat [E] / (Km + [S]).

25 Figures 12A and 12B contain two panels that illustrate the effect of variation to the length of the substrate binding regions of a DNA enzyme of the invention, as described in Example 6. The length of the complementary substrate binding region was varied in length (n) from 4 to

30 13 nucleotides for each the first and second substrate binding region (arm), as noted, and the catalytic activity was measured and expressed as k_{cat} (min^{-1}) and K_m (nanomolar [nM]).

Figure 13 illustrates the effect of modifications to
the nucleotide residues of a DNA enzyme, as described in
Example 6. DNA enzymes were incubated in 10%
heat-inactivated fetal bovine serum in RPMI-1640 media at 37
5   ⸬C, comparing unmodified DNA (open circles), inverted
thymidylate (filled circles), five 2'-O-Me residues in each
arm, (open squares), all 2'-O-Me residues in each arm
(filled squares), five P=S residues in core (open
triangles), and three P=S residues in each arm (filled
10   triangles).

DETAILED DESCRIPTION
     A.   Definitions
          As used herein, the term "deoxyribozyme" is used to
15   describe a DNA-containing nucleic acid that is capable of
functioning as an enzyme. In the present disclosure, the
term "deoxyribozyme" includes endoribonucleases and
endodeoxyribonucleases, although deoxyribozymes with
endoribonuclease activity are particularly preferred. Other
20   terms used interchangeably with deoxyribozyme herein are
"enzymatic DNA molecule", "DNAzyme", or "catalytic DNA
molecule", which terms should all be understood to include
enzymatically active portions thereof, whether they are
produced synthetically or derived from organisms or other
25   sources.
          The term "enzymatic DNA molecules" also includes DNA
molecules that have complementarity in a substrate-binding
region to a specified oligonucleotide target or substrate;
such molecules also have an enzymatic activity which is
30   active to specifically cleave the oligonucleotide substrate.
Stated in another fashion, the enzymatic DNA molecule is
capable of cleaving the oligonucleotide substrate
intermolecularly. This complementarity functions to allow

sufficient hybridization of the enzymatic DNA molecule to
the substrate oligonucleotide to allow the intermolecular
cleavage of the substrate to occur. While one-hundred
percent (100%) complementarity is preferred, complementarity

5   in the range of 75-100% is also useful and contemplated by
the present invention.

Enzymatic DNA molecules of the present invention may
alternatively be described as having nuclease or
ribonuclease activity. These terms may be used

10   interchangeably herein.

The term "enzymatic nucleic acid" as used herein
encompasses enzymatic RNA or DNA molecules, enzymatic RNA-
DNA polymers, and enzymatically active portions or
derivatives thereof, although enzymatic DNA molecules are a

15   particularly preferred class of enzymatically active
molecules according to the present invention.

The term "endodeoxyribonuclease", as used herein, is an
enzyme capable of cleaving a substrate comprised
predominantly of DNA. The term "endoribonuclease", as used

20   herein, is an enzyme capable of cleaving a substrate
comprised predominantly of RNA.

As used herein, the term "base pair" (bp) is generally
used to describe a partnership of adenine (A) with thymine
(T) or uracil (U), or of cytosine (C) with guanine (G),

25   although it should be appreciated that less-common analogs
of the bases A, T, C, and G (as well as U) may occasionally
participate in base pairings. Nucleotides that normally
pair up when DNA or RNA adopts a double stranded
configuration may also be referred to herein as

30   "complementary bases".

"Complementary nucleotide sequence" generally refers to
a sequence of nucleotides in a single-stranded molecule or
segment of DNA or RNA that is sufficiently complementary to

that on another single oligonucleotide strand to
specifically hybridize to it with consequent hydrogen
bonding.

"Nucleotide" generally refers to a monomeric unit of
DNA or RNA consisting of a sugar moiety (pentose), a
phosphate group, and a nitrogenous heterocyclic base. The
base is linked to the sugar moiety via the glycosidic carbon
(1' carbon of the pentose) and that combination of base and
sugar is a "nucleoside". When the nucleoside contains a
phosphate group bonded to the 3' or 5' position of the
pentose, it is referred to as a nucleotide. A sequence of
operatively linked nucleotides is typically referred to
herein as a "base sequence" or "nucleotide sequence", and
their grammatical equivalents, and is represented herein by
a formula whose left to right orientation is in the
conventional direction of 5'-terminus to 3'-terminus, unless
otherwise specified.

"Nucleotide analog" generally refers to a purine or
pyrimidine nucleotide that differs structurally from A, T,
G, C, or U, but is sufficiently similar to substitute for
the normal nucleotide in a nucleic acid molecule. As used
herein, the term "nucleotide analog" encompasses altered
bases, different or unusual sugars (i.e. sugars other than
the "usual" pentose), or a combination of the two. A
listing of exemplary analogs wherein the base has been
altered is provided in section C hereinbelow.

"Oligonucleotide or polynucleotide" generally refers to
a polymer of single- or double-stranded nucleotides. As
used herein, "oligonucleotide" and its grammatical
equivalents will include the full range of nucleic acids.
An oligonucleotide will typically refer to a nucleic acid
molecule comprised of a linear strand of ribonucleotides.
The exact size will depend on many factors, which in turn

depends on the ultimate conditions of use, as is well known
in the art.

As used herein, the term "physiologic conditions" is
meant to suggest reaction conditions emulating those found
5   in mammalian organisms, particularly humans. While
variables such as temperature, availability of cations, and
pH ranges may vary as described in greater detail below,
"physiologic conditions" generally comprise a temperature of
about 35-40°C, with 37°C being particularly preferred, as
10   well as a pH of about 7.0-8.0, with 7.5 being particularly
preferred, and further comprise the availability of cations,
preferably divalent and/or monovalent cations, with a
concentration of about 2-15 mM Mg^{2+} and 0-1.0 M Na+ being
particularly preferred. "Physiologic conditions", as used
15   herein, may optionally include the presence of free
nucleoside cofactor. As noted previously, preferred
conditions are described in greater detail below.

B.   Enzymatic DNA Molecules

20   In various embodiments, an enzymatic DNA molecule
of the present invention may combine one or more
modifications or mutations including additions, deletions,
and substitutions. In alternative embodiments, such
mutations or modifications may be generated using methods
25   which produce random or specific mutations or modifications.
These mutations may, for example, change the length of, or
alter the nucleotide sequence of, a loop, a spacer region or
the recognition sequence (or domain). One or more mutations
within one catalytically active enzymatic DNA molecule may
30   be combined with the mutation(s) within a second
catalytically active enzymatic DNA molecule to produce a new
enzymatic DNA molecule containing the mutations of both
molecules.

        In other preferred embodiments, an enzymatic DNA
molecule of the present invention may have random mutations
introduced into it using a variety of methods well known to
those skilled in the art. For example, the methods
5   described by Cadwell et al, <u>PCR Methods and Applications</u>,
2:28-33, 1992, are particularly preferred for use as
disclosed herein, with some modifications, as described in
the Examples that follow. (Also see Cadwell et al, <u>PCR
Methods and Applications</u>, 3(Suppl.):S136-S140, 1994.)
10 According to this modified PCR method, random point
mutations may be introduced into cloned genes.

        The aforementioned methods have been used, for example,
to mutagenize genes encoding ribozymes with a mutation rate
of 0.66% ± 0.13% (95% confidence interval) per position, as
15 determined by sequence analysis, with no strong preferences
observed with respect to the type of base substitution.
This allows the introduction of random mutations at any
position in the enzymatic DNA molecules of the present
invention.

20   Another method useful in introducing defined or random
mutations is disclosed in Joyce et al, <u>Nucleic Acids Res.</u>,
17:711-722, 1989. This latter method involves excision of a
template (coding) strand of a double-stranded DNA,
reconstruction of the template strand with inclusion of
25 mutagenic oligonucleotides, and subsequent transcription of
the partially-mismatched template. This allows the
introduction of defined or random mutations at any position
in the molecule by including polynucleotides containing
known or random nucleotide sequences at selected positions.

30   Enzymatic DNA molecules of the present invention may be
of varying lengths and folding patterns, as appropriate,
depending on the type and function of the molecule. For
example, enzymatic DNA molecules may be about 15 to about

400 or more nucleotides in length, although a length not
exceeding about 250 nucleotides is preferred, to avoid
limiting the therapeutic usefulness of molecules by making
them too large or unwieldy. In various preferred

5   embodiments, an enzymatic DNA molecule of the present
invention is at least about 20 nucleotides in length and,
while useful molecules may exceed 100 nucleotides in length,
preferred molecules are generally not more than about 100
nucleotides in length.

10   In various therapeutic applications, enzymatic DNA
molecules of the present invention comprise the
enzymatically active portions of deoxyribozymes. In various
embodiments, enzymatic DNA molecules of the present
invention preferably comprise not more than about 200

15   nucleotides. In other embodiments, a deoxyribozyme of the
present invention comprises not more than about 100
nucleotides. In still other preferred embodiments,
deoxyribozymes of the present invention are about 20-75
nucleotides in length, more preferably about 20-65

20   nucleotides in length. Other preferred enzymatic DNA
molecules are about 10-50 nucleotides in length.

            In other applications, enzymatic DNA molecules may
assume configurations similar to those of "hammerhead"
ribozymes. Such enzymatic DNA molecules are preferably no

25   more than about 75-100 nucleotides in length, with a length
of about 20-50 nucleotides being particularly preferred.

            In general, if one intends to synthesize molecules for
use as disclosed herein, the larger the enzymatic nucleic
acid molecule is, the more difficult it is to synthesize.

30   Those of skill in the art will certainly appreciate these
design constraints. Nevertheless, such larger molecules
remain within the scope of the present invention.

            It is also to be understood that an enzymatic DNA

molecule of the present invention may comprise enzymatically
active portions of a deoxyribozyme or may comprise a
deoxyribozyme with one or more mutations, e.g., with one or
more base-pair-forming sequences or spacers absent or
5   modified, as long as such deletions, additions or
modifications do not adversely impact the molecule's ability
to perform as an enzyme.

The recognition domain of an enzymatic DNA molecule of
the present invention typically comprises two nucleotide
10   sequences flanking a catalytic domain, and typically
contains a sequence of at least about 3 to about 30 bases,
preferably about 6 to about 15 bases, which are capable of
hybridizing to a complementary sequence of bases within the
substrate nucleic acid giving the enzymatic DNA molecule its
15   high sequence specificity. Modification or mutation of the
recognition site via well-known methods allows one to alter
the sequence specificity of an enzymatic nucleic acid
molecule. (See Joyce et al, Nucleic Acids Res., 17:711-712,
1989.)

20   Enzymatic nucleic acid molecules of the present
invention also include those with altered recognition sites
or domains. In various embodiments, these altered
recognition domains confer unique sequence specificities on
the enzymatic nucleic acid molecule including such
25   recognition domains. The exact bases present in the
recognition domain determine the base sequence at which
cleavage will take place. Cleavage of the substrate nucleic
acid occurs within the recognition domain. This cleavage
leaves a 2', 3', or 2',3'-cyclic phosphate group on the
30   substrate cleavage sequence and a 5' hydroxyl on the
nucleotide that was originally immediately 3' of the
substrate cleavage sequence in the original substrate.
Cleavage can be redirected to a site of choice by changing

-28-

the bases present in the recognition sequence (internal guide sequence). See Murphy et al, <u>Proc. Natl. Acad. Sci. USA</u>, 86:9218-9222, 1989.

Moreover, it may be useful to add a polyamine to facilitate recognition and binding between the enzymatic DNA molecule and its substrate. Examples of useful polyamines include spermidine, putrescine or spermine. A spermidine concentration of about 1 mM may be effective in particular embodiments, while concentrations ranging from about 0.1 mM to about 10 mM may also be useful.

In various alternative embodiments, an enzymatic DNA molecule of the present invention has an enhanced or optimized ability to cleave nucleic acid substrates, preferably RNA substrates. As those of skill in the art will appreciate, the rate of an enzyme-catalyzed reaction varies depending upon the substrate and enzyme concentrations and, in general, levels off at high substrate or enzyme concentrations. Taking such effects into account, the kinetics of an enzyme-catalyzed reaction may be described in the following terms, which define the reaction.

The enhanced or optimized ability of an enzymatic DNA molecule of the present invention to cleave an RNA substrate may be determined in a cleavage reaction with varying amounts of labeled RNA substrate in the presence of enzymatic DNA molecule. The ability to cleave the substrate is generally defined by the catalytic rate (k_{cat}) divided by the Michaelis constant (K_M). The symbol k_{cat} represents the maximal velocity of an enzyme reaction when the substrate approaches a saturation value. K_M represents the substrate concentration at which the reaction rate is one-half maximal.

For example, values for K_M and k_{cat} may be determined in this invention by experiments in which the substrate

concentration [S] is in excess over enzymatic DNA molecule
concentration [E]. Initial rates of reaction (v_o) over a
range of substrate concentrations are estimated from the
initial linear phase, generally the first 5% or less of the
reaction. Data points are fit by a least squares method to
a theoretical line given by the equation: $v = -K_M(v_o/[S]) + V_{max}$. Thus, k_{cat} and K_M are determined by the initial rate of
reaction, v_o, and the substrate concentration [S].

In various alternative embodiments, an enzymatic DNA
molecule of the present invention has an enhanced or
optimized ability to cleave nucleic acid substrates,
preferably RNA substrates. In preferred embodiments, the
enhanced or optimized ability of an enzymatic DNA molecule
to cleave RNA substrates shows about a 10- to 10^9-fold
improvement over the uncatalyzed rate. In more preferred
embodiments, an enzymatic DNA molecule of the present
invention is able to cleave RNA substrates at a rate that is
about 10^3- to 10^7-fold improved over "progenitor" species.
In even more preferred embodiments, the enhanced or
optimized ability to cleave RNA substrates is expressed as a
10^4- to 10^6-fold improvement over the progenitor species.
One skilled in the art will appreciate that the enhanced or
optimized ability of an enzymatic DNA molecule to cleave
nucleic acid substrates may vary depending upon the
selection constraints applied during the *in vitro* evolution
procedure of the invention.

Various preferred methods of modifying deoxyribozymes
and other enzymatic DNA molecules and nucleases of the
present invention are further described in Examples 1-3
hereinbelow.

C.   Nucleotide Analogs

As noted above, the term "nucleotide analog" as

used herein generally refers to a purine or pyrimidine
nucleotide that differs structurally from A, T, G, C, or U,
but is sufficiently similar to substitute for such "normal"
nucleotides in a nucleic acid molecule. As used herein, the
term "nucleotide analog" encompasses altered bases,
different (or unusual) sugars, altered phosphate backbones,
or any combination of these alterations. Examples of
nucleotide analogs useful according to the present invention
include those listed in the following Table, most of which
are found in the approved listing of modified bases at 37
CFR §1.822 (which is incorporated herein by reference).

Table 1
Nucleotide Analogs

Abbreviation	Description
ac4c	4-acetylcytidine
chm5u	5-(carboxyhydroxylmethyl)uridine
cm	2'-O-methylcytidine
cmnm5s2u	5-carboxymethylaminomethyl-2-thiouridine
d	dihydrouridine
fm	2'-O-methylpseudouridine
galq	ß, D-galactosylqueosine
gm	2'-O-methylguanosine
I	inosine
i6a	N6-isopentenyladenosine
m1a	1-methyladenosine
m1f	1-methylpseudouridine
m1g	1-methylguanosine
m1l	1-methylinosine
m22g	2,2-dimethylguanosine
m2a	2-methyladenosine

	m2g	2-methylguanosine
	m3c	3-methylcytidine
	m5c	5-methylcytidine
	m6a	N6-methyladenosine
5	m7g	7-methylguanosine
	mam5u	5-methylaminomethyluridine
	mam5s2u	5-methoxyaminomethyl-2-thiouridine
	manq	ß, D-mannosylmethyluridine
	mcm5s2u	5-methoxycarbonylmethyluridine
10	mo5u	5-methoxyuridine
	ms2i6a	2-methylthio-N6-isopentenyladenosine
	ms2t6a	N-((9-ß-D-ribofuranosyl-2-methyl thiopurine-6-yl)carbamoyl)threonine
15	mt6a	N-((9-ß-D-ribofuranosylpurine-6-yl)N-methyl-carbamoyl)threonine
	mv	uridine-5-oxyacetic acid methylester
	o5u	uridine-5-oxyacetic acid (v)
	osyw	wybutoxosine
	p	pseudouridine
20	q	queosine
	s2c	2-thiocytidine
	s2t	5-methyl-2-thiouridine
	s2u	2-thiouridine
	s4u	4-thiouridine
25	t	5-methyluridine
	t6a	N-((9-ß-D-ribofuranosylpurine-6-yl)carbamoyl)threonine
	tm	2'-O-methyl-5-methyluridine
	um	2'-O-methyluridine
30	yw	wybutosine
	x	3-(3-amino-3-carboxypropyl)uridine, (acp3)u
	araU	ß, D-arabinosyl

araT   ß, D-arabinosyl

5   Other useful analogs include those described in
published international application no. WO 92/20823 (the
disclosures of which are incorporated herein by reference),
or analogs made according to the methods disclosed therein.
Analogs described in DeMesmaeker et al, _Angew. Chem. Int._
10 _Ed. Engl.,_ 33:226-229, 1994; DeMesmaeker et al, _Synlett_
733-736 (Oct. 1993); Nielsen et al, _Science,_ 254:1497-1500,
1991; and Idziak et al, _Tetrahedron Letters,_ 34:5417-5420,
1993, are also useful according to the within-disclosed
invention and said disclosures are incorporated by reference
15 herein.

      D.   Methods of Engineering Enzymatic DNA Molecules
       The present invention also contemplates methods of
producing nucleic acid molecules having a predetermined
20 activity. In one preferred embodiment, the nucleic acid
molecule is an enzymatic DNA molecule. In another
variation, the desired activity is a catalytic activity.
       In one embodiment, the present invention contemplates
methods of synthesizing enzymatic DNA molecules that may
25 then be "engineered" to catalyze a specific or predetermined
reaction. Methods of preparing enzymatic DNA molecules are
described herein; see, e.g., Examples 1-3 hereinbelow. In
other embodiments, an enzymatic DNA molecule of the present
invention may be engineered to bind small molecules or
30 ligands, such as adenosine triphosphate (ATP). (See
Sassanfar et al, _Nature,_ 364:550-553, 1993.)
       In another embodiment, the present invention
contemplates that a population of enzymatic DNA molecules
may be subjected to mutagenizing conditions to produce a
35 diverse population of mutant enzymatic DNA molecules (which

may alternatively be called "deoxyribozymes" or "DNAzymes").
Thereafter, enzymatic DNA molecules having desired
characteristics are selected and/or separated from the
population and are subsequently amplified.

5   Alternatively, mutations may be introduced in the
enzymatic DNA molecule by altering the length of the
recognition domains of the enzymatic DNA molecule. The
recognition domains of the enzymatic DNA molecule associate
with a complementary sequence of bases within a substrate
10   nucleic acid sequence. Methods of altering the length of
the recognition domains are known in the art and include
PCR, for example; useful techniques are described further in
the Examples below.

          Alteration of the length of the recognition domains of
15   an enzymatic DNA molecule may have a desirable effect on the
binding specificity of the enzymatic DNA molecule. For
example, an increase in the length of the recognition
domains may increase binding specificity between the
enzymatic DNA molecule and the complementary base sequences
20   of an oligonucleotide in a substrate, or may enhance
recognition of a particular sequence in a hybrid substrate.
In addition, an increase in the length of the recognition
domains may also increase the affinity with which it binds
to substrate. In various embodiments, these altered
25   recognition domains in the enzymatic DNA molecule confer
increased binding specificity and affinity between the
enzymatic DNA molecule and its substrate.

          It has recently been noted that certain
oligonucleotides are able to recognize and bind molecules
30   other than oligonucleotides with complementary sequences.
These oligonucleotides are often given the name "aptamers".
For example, Ellington et al describe RNA molecules that are
able to bind a variety of organic dyes (Nature, 346:818-822,

1990), while Bock et al describe ssDNA molecules that bind
human thrombin (Nature, 355:564-566, 1992). Similarly,
Jellinek et al describe RNA ligands to basic fibroblast
growth factor (Proc.Natl.Acad.Sci. USA, 90:11227-11231,
1993). Thus, it is further contemplated herein that the
catalytically active DNA enzymes of the present invention
may be engineered according to the within-described methods
to display a variety of capabilities typically associated
with aptamers.

One of skill in the art should thus appreciate that the
enzymatic DNA molecules of this invention can be altered at
any nucleotide sequence, such as the recognition domains, by
various methods disclosed herein, including PCR and 3SR
(self-sustained sequence replication -- see Example 1
below). For example, additional nucleotides can be added to
the 5' end of the enzymatic DNA molecule by including
additional nucleotides in the primers.

Enzymatic DNA molecules of the present invention may
also be prepared or engineered in a more non-random fashion
via use of methods such as site-directed mutagenesis. For
example, site-directed mutagenesis may be carried out
essentially as described in Morinaga et al, Biotechnology,
2:636, 1984, modified as described herein, for application
to deoxyribozymes. Useful methods of engineering enzymatic
DNA molecules are further described in the Examples below.

In one disclosed embodiment, an enzymatic DNA molecule
of the present invention comprises a conserved core flanked
by two substrate binding (or recognition) domains or
sequences that interact with the substrate through
base-pairing interactions. In various embodiments, the
conserved core comprises one or more conserved domains or
sequences. In another variation, an enzymatic DNA molecule
further comprises a "spacer" region (or sequence) between

the regions (or sequences) involved in base pairing. In
still another variation, the conserved core is "interrupted"
at various intervals by one or more less-conserved variable
or "spacer" nucleotides.

5   In various embodiments, the population of enzymatic DNA
molecules is made up of at least 2 different types of
deoxyribozyme molecules. For example, in one variation, the
molecules have differing sequences. In another variation,
the deoxyribozymes are nucleic acid molecules having a

10   nucleic acid sequence defining a recognition domain that is
contiguous or adjacent to the 5'-terminus of the nucleotide
sequence. In various alternative embodiments, enzymatic DNA
molecules of the present invention may further comprise one
or more spacer regions located 3'-terminal to the

15   recognition domains, one or more loops located 3'-terminal
to the recognition domains and/or spacer regions. In other
variations, a deoxyribozyme of the present invention may
comprise one or more regions which are capable of
hybridizing to other regions of the same molecule. Other

20   characteristics of enzymatic DNA molecules produced
according to the presently-disclosed methods are described
elsewhere herein.

In other embodiments, mutagenizing conditions include
conditions that introduce either defined or random

25   nucleotide substitutions within an enzymatic DNA molecule.
Examples of typical mutagenizing conditions include
conditions disclosed in other parts of this specification
and the methods described by Joyce et al, Nucl. Acids Res.,
17:711-722, 1989; Joyce, Gene, 82:83-87, 1989; and Beaudry

30   et al, Science, 257:635-41, 1992.

In still other embodiments, a diverse population of
mutant enzymatic nucleic acid molecules of the present
invention is one that contains at least 2 nucleic acid

molecules that do not have the exact same nucleotide
sequence.   In other variations, from such a diverse
population, an enzymatic DNA molecule or other enzymatic
nucleic acid having a predetermined activity is then
selected on the basis of its ability to perform the
predetermined activity.   In various embodiments, the
predetermined activity comprises, without limitation,
enhanced catalytic activity, decreased K_M, enhanced
substrate binding ability, altered substrate specificity,
and the like.

Other parameters which may be considered aspects of
enzyme performance include catalytic activity or capacity,
substrate binding ability, enzyme turnover rate, enzyme
sensitivity to feedback mechanisms, and the like.   In
certain aspects, substrate specificity may be considered an
aspect of enzyme performance, particularly in situations in
which an enzyme is able to recognize and bind two or more
competing substrates, each of which affects the enzyme's
performance with respect to the other substrate(s).

Substrate specificity, as used herein, may refer to the
specificity of an enzymatic nucleic acid molecule as
described herein for a particular substrate, such as one
comprising ribonucleotides only, deoxyribonucleotides only,
or a composite of both.   Substrate molecules may also
contain nucleotide analogs.   In various embodiments, an
enzymatic nucleic acid molecule of the present invention may
preferentially bind to a particular region of a hybrid or
non-hybrid substrate.

The term or parameter identified herein as "substrate
specificity" may also include sequence specificity; i.e., an
enzymatic nucleic acid molecule of the present invention may
"recognize" and bind to a nucleic acid substrate having a
particular nucleic acid sequence.   For example, if the

substrate recognition domains of an enzymatic nucleic acid
molecule of the present invention will only bind to
substrate molecules having a series of one or two
ribonucleotides (e.g., rA) in a row, then the enzymatic

5   nucleic acid molecule will tend not to recognize or bind
nucleic acid substrate molecules lacking such a sequence.

With regard to the selection process, in various
embodiments, selecting includes any means of physically
separating the mutant enzymatic nucleic acids having a

10   predetermined activity from the diverse population of mutant
enzymatic nucleic acids. Often, selecting comprises
separation by size, by the presence of a catalytic activity,
or by hybridizing the mutant nucleic acid to another nucleic
acid, to a peptide, or some other molecule that is either in

15   solution or attached to a solid matrix.

In various embodiments, the predetermined activity is
such that the mutant enzymatic nucleic acid having the
predetermined activity becomes labeled in some fashion by
virtue of the activity. For example, the predetermined

20   activity may be an enzymatic DNA molecule activity whereby
the activity of the mutant enzymatic nucleic acid upon its
substrate causes the mutant enzymatic nucleic acid to become
covalently linked to it. The mutant enzymatic nucleic acid
is then selected by virtue of the covalent linkage.

25   In other embodiments, selecting a mutant enzymatic
nucleic acid having a predetermined activity includes
amplification of the mutant enzymatic nucleic acid (see
Joyce, Gene, 82:83-87, 1989; Beaudry et al, Science,
257:635-41, 1992). Other methods of selecting an enzymatic

30   nucleic acid molecule having a predetermined characteristic
or activity are described in the Examples section.

E.   Compositions

The invention also contemplates compositions
containing one or more types or populations of enzymatic DNA
molecules of the present invention; e.g., different types or
populations may recognize and cleave different nucleotide
5   sequences. Compositions may further include a ribonucleic
acid-containing substrate. Compositions according to the
present invention may further comprise lead ion, magnesium
ion, or other divalent or monovalent cations, as discussed
herein.

10   Preferably, the enzymatic DNA molecule is present at a
concentration of about 0.05 μM to about 2 μM. Typically,
the enzymatic DNA molecule is present at a concentration
ratio of enzymatic DNA molecule to substrate of from about
1:5 to about 1:50. More preferably, the enzymatic DNA
15   molecule is present in the composition at a concentration of
about 0.1 μM to about 1 μM. Even more preferably,
compositions contain the enzymatic DNA molecule at a
concentration of about 0.1 μM to about 0.5 μM. Preferably,
the substrate is present in the composition at a
20   concentration of about 0.5 μM to about 1000 μM.

One skilled in the art will understand that there are
many sources of nucleic acid-containing substrates including
naturally-occurring and synthetic sources. Sources of
suitable substrates include, without limitation, a variety
25   of viral and retroviral agents, including HIV-1, HIV-2,
HTLV-I, and HTLV-II.

Other suitable substrates include, without limitation,
viral and retroviral agents including those comprising or
produced by picornaviruses, hepadnaviridae (e.g., HBV, HCV),
30   papillomaviruses (e.g., HPV), gammaherpesvirinae (e.g.,
EBV), lymphocryptoviruses, leukemia viruses (e.g., HTLV-I
and -II), flaviviruses, togaviruses, herpesviruses
(including alphaherpesviruses and betaherpesviruses),

cytomegaloviruses (CMV), influenza viruses, and viruses and
retroviruses contributing to immunodeficiency diseases and
syndromes (e.g., HIV-1 and -2). In addition, suitable
substrates include viral and retroviral agents which infect
5   non-human primates and other animals including, without
limitation, the simian and feline immunodeficiency viruses
and bovine leukemia viruses.

Magnesium ion, lead ion, or another suitable monovalent
or divalent cation, as described previously, may also be
10   present in the composition, at a concentration ranging from
about 1-100 mM. More preferably, the preselected ion is
present in the composition at a concentration of about 2 mM
to about 50 mM, with a concentration of about 5 mM being
particularly preferred. One skilled in the art will
15   understand that the ion concentration is only constrained by
the limits of solubility of its source (e.g. magnesium) in
aqueous solution and a desire to have the enzymatic DNA
molecule present in the same composition in an active
conformation.

20   The invention also contemplates compositions containing
an enzymatic DNA molecule of the present invention, hybrid
deoxyribonucleotide-ribonucleotide molecules, and magnesium
or lead ion in concentrations as described hereinabove. As
noted previously, other monovalent or divalent ions (e.g.,
25   Ca^{2+}) may be used in place of magnesium.

Also contemplated by the present invention are
compositions containing an enzymatic DNA molecule of the
present invention, nucleic acid-containing substrate (e.g.
RNA), and a preselected ion at a concentration of greater
30   than about 1 millimolar, wherein said substrate is greater
in length than the recognition domains present on the
enzymatic DNA molecule.

In one variation, a composition comprises an enzymatic

DNA molecule-substrate complex, wherein base pairing between an enzymatic DNA molecule and its substrate is contiguous. In another embodiment, base pairing between an enzymatic DNA molecule and its substrate is interrupted by one or more

5   noncomplementary pairs. In a variety of alternative embodiments, a composition of the present invention may further comprise a monovalent cation, a divalent cation, or both.

In another variation, an enzymatic DNA molecule of the

10   present invention is capable of functioning efficiently in the presence or absence of a divalent cation. In one variation, a divalent cation is present and comprises Pb^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, or Ca^{2+}. Alternatively, an enzymatic DNA molecule of the present invention is capable of functioning

15   efficiently in the presence or absence of monovalent cations. It is anticipated that monovalent or divalent cation concentrations similar to those described herein for Pb^{2+} or Mg^{2+} will be useful as disclosed herein.

Optionally, monovalent cations may also be present in

20   addition to, or as "alternatives" for, divalent cations. For example, monovalent cations such as sodium (Na^+) or potassium (K^+) may be present, either as dissociated ions or in the form of dissociable compounds such as NaCl or KCl.

In one embodiment, the concentration of monovalent

25   cation present in the composition ranges from 0 - 1.0 M. In another embodiment, a monovalent cation is present in a concentration ranging from about 0-200 mM. In other embodiments, monovalent cations are present in a concentration ranging from about 1-100 mM. Alternatively,

30   the concentration of monovalent cations ranges from about 2 mM - 50 mM. In still other embodiments, the concentration ranges from about 2 mM - 25 mM.

F.   Methods of Using Enzymatic DNA Molecules

Methods for using enzymatic DNA molecules as disclosed herein cover many different utilities as are well known in the art for prior art enzymatic and/or antisense oligonucleotides. As discussed previously, molecules capable of cleaving the bonds linking neighboring nucleic acids (e.g., phosphoester bonds) have numerous uses encompassing a wide variety of applications. For example, enzymatic DNA molecules having the within-disclosed capabilities, structures, and/or functions are useful in pharmaceutical and medical products (e.g., for wound debridement, clot dissolution, etc.), as well as in household items (e.g., detergents, dental hygiene products, meat tenderizers). For inactivating target nucleic acid sequences, such as mRNA in vitro and in vivo. Industrial utility of the within-disclosed compounds, compositions and methods is also contemplated and well within the scope of the present invention.

The present invention also describes useful methods for cleaving any single-stranded, looped, partially or fully double-stranded nucleic acid; the majority of these methods employ the novel enzymatically active nucleic acid molecules of the present invention. In various embodiments, the single-stranded nucleic acid segment or portion of the substrate (or the entire substrate itself) comprises DNA, modified DNA, RNA, modified RNA, or composites thereof. Preferably, the nucleic acid substrate need only be single-stranded at or near the substrate cleavage sequence so that an enzymatic nucleic acid molecule of the present invention can hybridize to the substrate cleavage sequence by virtue of the enzyme's recognition sequence.

A nucleic acid substrate that can be cleaved by a method of this invention may be chemically synthesized or

-42-

enzymatically produced, or it may be isolated from various
sources such as phages, viruses, prokaryotic cells, or
eukaryotic cells, including animal cells, plant cells, yeast
cells and bacterial cells. Chemically synthesized single-

5   and double-stranded nucleic acids are commercially available
from many sources including, without limitation, Research
Genetics (Huntsville, AL).

RNA substrates may also be synthesized using an Applied
Biosystems (Foster City, CA) oligonucleotide synthesizer

10   according to the manufacturer's instructions. Single-
stranded phage are also a source of nucleic acid substrates.
(See Messing et al, Proc.Natl.Acad.Sci. USA, 74:3642-3646,
1977, and Yanisch-Perron et al, Gene, 33:103-119, 1985).
Bacterial cells containing single-stranded phage would also

15   be a ready source of suitable single-stranded nucleic acid
substrates.

Single-stranded RNA cleavable by a method of the
present invention could be provided by any of the RNA
viruses such as the picornaviruses, togaviruses,

20   orthomyxoviruses, paramyxoviruses, rhabdoviruses,
coronaviruses, arenaviruses or retroviruses. As noted
previously, a wide variety of prokaryotic and eukaryotic
cells may also be excellent sources of suitable nucleic acid
substrates.

25   The methods of this invention may be used on single-
stranded nucleic acids or single-stranded portions of looped
or double-stranded nucleic acids that are present inside a
cell, including eukaryotic, procaryotic, plant, animal,
yeast or bacterial cells. Under these conditions an

30   enzymatic nucleic acid molecule (e.g., an enzymatic DNA
molecule or deoxyribozyme) of the present invention could
act as an anti-viral agent or a regulator of gene
expression. Examples of such uses of enzymatic DNA

molecules of the present invention are described further hereinbelow.

In the majority of methods of the present invention, cleavage of single-stranded nucleic acids occurs at the 3'-terminus of a predetermined base sequence. This predetermined base sequence or substrate cleavage sequence typically contains from 1 to about 10 nucleotides. In other preferred embodiments, an enzymatic DNA molecule of the present invention is able to recognize nucleotides either upstream, or upstream *and* downstream of the cleavage site. In various embodiments, an enzymatic DNA molecule is able to recognize about 2-10 nucleotides upstream of the cleavage site; in other embodiments, an enzymatic DNA molecule is able to recognize about 2-10 nucleotides upstream and about 2-10 nucleotides downstream of the cleavage site. Other preferred embodiments contemplate an enzymatic DNA molecule that is capable of recognizing a nucleotide sequence up to about 30 nucleotides in length, with a length up to about 20 nucleotides being even more preferred.

The within-disclosed methods allow cleavage at any nucleotide sequence by altering the nucleotide sequence of the recognition domains of the enzymatic DNA molecule. This allows cleavage of single-stranded nucleic acid in the absence of a restriction endonuclease site at the selected position.

An enzymatic DNA molecule of the present invention may be separated from any portion of the single-stranded nucleic acid substrate that remains attached to the enzymatic DNA molecule by site-specific hydrolysis at the appropriate cleavage site. Separation of the enzymatic DNA molecule from the substrate (or "cleavage product") allows the enzymatic DNA molecule to carry out another cleavage reaction.

Generally, the nucleic acid substrate is treated under appropriate nucleic acid cleaving conditions -- preferably, physiologic conditions -- with an effective amount of an enzymatic DNA molecule of the present invention. If the

5   nucleic acid substrate comprises DNA, cleaving conditions may include the presence of a divalent cation at a concentration of about 2-10 mM.

An effective amount of an enzymatic DNA molecule is the amount required to cleave a predetermined base sequence

10   present within the single-stranded nucleic acid. Preferably, the enzymatic DNA molecule is present at a molar ratio of DNA molecule to substrate cleavage sites of 1 to 20. This ratio may vary depending on the length of treating and efficiency of the particular enzymatic DNA molecule

15   under the particular nucleic acid cleavage conditions employed.

Thus, in one preferred embodiment, treating typically involves admixing, in aqueous solution, the RNA-containing substrate and the enzyme to form a cleavage admixture, and

20   then maintaining the admixture thus formed under RNA cleaving conditions for a time period sufficient for the enzymatic DNA molecule to cleave the RNA substrate at any of the predetermined nucleotide sequences present in the RNA. In various embodiments, a source of ions is also provided --

25   i.e. monovalent or divalent cations, or both.

In one embodiment of the present invention, the amount of time necessary for the enzymatic DNA molecule to cleave the single-stranded nucleic acid has been predetermined. The amount of time is from about 1 minute to about 24 hours

30   and will vary depending upon the concentration of the reactants and the temperature of the reaction. Usually, this time period is from about 10 minutes to about 2 hours such that the enzymatic DNA molecule cleaves the single-

stranded nucleic acid at any of the predetermined nucleotide
sequences present.

The invention further contemplates that the nucleic
acid cleaving conditions include the presence of a source of
5   divalent cations (e.g., PbOAc) at a concentration of about
2-100 mM. Typically, the nucleic acid cleaving conditions
include divalent cation at a concentration of about 2 mM to
about 10 mM, with a concentration of about 5 mM being
particularly preferred.

10   The optimal cationic concentration to include in the
nucleic acid cleaving conditions can be easily determined by
determining the amount of single-stranded nucleic acid
cleaved at a given cation concentration. One skilled in the
art will understand that the optimal concentration may vary
15   depending on the particular enzymatic DNA molecule employed.

The present invention further contemplates that the
nucleic acid cleaving conditions include a pH of about pH
6.0 to about pH 9.0. In one preferred embodiment, the pH
ranges from about pH 6.5 to pH 8.0. In another preferred
20   embodiment, the pH emulates physiological conditions, i.e.,
the pH is about 7.0-7.8, with a pH of about 7.5 being
particularly preferred.

One skilled in the art will appreciate that the methods
of the present invention will work over a wide pH range so
25   long as the pH used for nucleic acid cleaving is such that
the enzymatic DNA molecule is able to remain in an active
conformation. An enzymatic DNA molecule in an active
conformation is easily detected by its ability to cleave
single-stranded nucleic acid at a predetermined nucleotide
30   sequence.

In various embodiments, the nucleic acid cleaving
conditions also include a variety of temperature ranges. As
noted previously, temperature ranges consistent with

physiological conditions are especially preferred, although temperature ranges consistent with industrial applications are also contemplated herein. In one embodiment, the temperature ranges from about 15°C to about 60°C. In

5   another variation, the nucleic acid cleaving conditions include a temperature ranging from about 30°C to about 56°C. In yet another variation, nucleic acid cleavage conditions include a temperature from about 35°C to about 50°C. In a preferred embodiment, nucleic acid cleavage conditions

10   comprise a temperature range of about 37°C to about 42°C. The temperature ranges consistent with nucleic acid cleaving conditions are constrained only by the desired cleavage rate and the stability of that particular enzymatic DNA molecule at that particular temperature.

15   In various methods, the present invention contemplates nucleic acid cleaving conditions including the presence of a polyamine. Polyamines useful for practicing the present invention include spermidine, putrescine, spermine and the like. In one variation, the polyamine is present at a

20   concentration of about .01 mM to about 10 mM. In another variation, the polyamine is present at a concentration of about 1 mM to about 10 mM. Nucleic acid cleavage conditions may also include the presence of polyamine at a concentration of about 2 mM to about 5 mM. In various

25   preferred embodiments, the polyamine is spermidine.

     G.   Vectors
           The present invention also features expression
     vectors including a nucleic acid segment encoding an

30   enzymatic DNA molecule of the present invention situated within the vector, preferably in a manner which allows expression of that enzymatic DNA molecule within a target cell (e.g., a plant or animal cell).

Thus, in general, a vector according to the present invention preferably includes a plasmid, cosmid, phagemid, virus, or phage vector. Preferably, suitable vectors comprise single-stranded DNA (ssDNA) -- e.g., circular phagemid ssDNA. It should also be appreciated that useful vectors according to the present invention need not be circular.

In one variation, nucleotide sequences flanking each of the additional enzymatic DNA molecule-encoding sequences are preferably provided, which sequences may be recognized by the first enzymatic DNA molecule. The intervening or flanking sequences preferably comprise at least 1 nucleotide; more preferably, intervening or flanking sequences are about 2-20 nucleotides in length, with sequences of about 5-10 nucleotides in length being particularly preferred.

The addition of polynucleotide tails may also be useful to protect the 3' end of an enzymatic DNA molecule according to the present invention. These may be provided by attaching a polymeric sequence by employing the enzyme terminal transferase.

A vector according to the present invention includes two or more enzymatic DNA molecules. In one embodiment, a first enzymatic DNA molecule has intramolecular cleaving activity and is able to recognize and cleave nucleotide sequences to release other enzymatic DNA sequences; i.e., it is able to function to "release" other enzymatic DNA molecules from the vector. For example, a vector is preferably constructed so that when the first enzymatic DNA molecule is expressed, that first molecule is able to cleave nucleotide sequences flanking additional nucleotide sequences encoding a second enzymatic DNA molecule, a third enzymatic DNA molecule, and so forth. Presuming said first

enzymatic DNA molecule (i.e., the "releasing" molecule) is
able to cleave oligonucleotide sequences intramolecularly,
the additional (e.g. second, third, and so on) enzymatic DNA
molecules (i.e., the "released" molecules) need not possess

5   characteristics identical to the "releasing" molecule. For
example, in one embodiment, the "released" (i.e., the
second, third, etc.) enzymatic DNA molecules are able to
cleave specific RNA sequences, while the first ("releasing")
enzymatic DNA molecule has nuclease activity allowing it to

10   liberate the "released" molecules. In another embodiment,
the "released" enzymatic DNA molecule has amide bond-
cleaving activity, while the first ("releasing") enzymatic
DNA molecule has nuclease activity.

       Alternatively, the first enzymatic DNA molecule may be

15   encoded on a separate vector from the second (and third,
fourth, etc.) enzymatic DNA molecule(s) and may have
intermolecular cleaving activity. As noted herein, the
first enzymatic DNA molecule can be a self-cleaving
enzymatic DNA molecule (e.g., a deoxyribozyme), and the

20   second enzymatic DNA molecule may be any desired type of
enzymatic DNA molecule. When a vector is caused to express
DNA from these nucleic acid sequences, that DNA has the
ability under appropriate conditions to cleave each of the
flanking regions, thereby releasing one or more copies of

25   the second enzymatic DNA molecule. If desired, several
different second enzymatic DNA molecules can be placed in
the same cell or carrier to produce different
deoxyribozymes. It is also contemplated that any one or
more vectors may comprise one or more ribozymes or

30   deoxyribozymes in any combination of "releasing" and
"released" enzymatic nucleic acid molecules, as long as such
a combination achieves the desired result: the release of
enzymatic nucleic acid molecules that are capable of

cleaving predetermined nucleic acid sequences.

Methods of isolating and purifying enzymatic DNA molecules of the present invention are also contemplated. In addition to the methods described herein, various

5   purification methods (e.g. those using HPLC) and chromatographic isolation techniques are available in the art. See, e.g., the methods described in published international application no. WO 93/23569, the disclosures of which are incorporated herein by reference.

10   It should also be understood that various combinations of the embodiments described herein are included within the scope of the present invention. Other features and advantages of the present invention will be apparent from the descriptions hereinabove, from the Examples to follow,

15   and from the claims.

EXAMPLES

The following examples illustrate, but do not limit, the present invention.

20

1.   In Vitro Evolution of Enzymatic DNA Molecules:An
         Overview

In vitro selection and *in vitro* evolution techniques allow new catalysts to be isolated without *a*

25   *priori* knowledge of their composition or structure. Such methods have been used to obtain RNA enzymes with novel catalytic properties. For example, ribozymes that undergo autolytic cleavage with lead cation have been derived from a randomized pool of tRNA^{Phe} molecules (Pan et al,

30   Biochemistry, 31:3887-3895, 1992). Group I ribozyme variants have been isolated that can cleave DNA (Beaudry et al, Science, 257:635-641, 1992) or that have altered metal dependence (Lehman et al, Nature, 361:182-185, 1993).

Starting with a pool of random RNA sequences, molecules have
been obtained that catalyze a polymerase-like reaction
(Bartel et al, Science, 261:1411-1418, 1993). In the
present example, refinement of specific catalytic properties
of an evolved enzyme via alteration of the selection
constraints during an *in vitro* evolution procedure is
described.

Darwinian evolution requires the repeated operation of
three processes: (a) introduction of genetic variation; (b)
selection of individuals on the basis of some fitness
criterion; and (c) amplification of the selected
individuals. Each of these processes can be realized *in*
vitro (Joyce, Gene, 82:83, 1989). A gene can be mutagenized
by chemical modification, incorporation of randomized
mutagenic oligodeoxynucleotides, or inaccurate copying by a
polymerase. (See Cadwell et al, PCR Methods and
Applications, 2:28-33, 1992); Cadwell et al, PCR Methods
and Applications, 3(Suppl.):S136-S140, 1994; Chu et al,
Virology, 98:168, 1979; Shortle et al, Meth. Enzymol.,
100:457, 1983; Myers et al, Science, 229:242, 1985;
Matteucci et al, Nucleic Acids Res., 11:3113, 1983; Wells
et al, Gene, 34:315, 1985; McNeil et al, Mol. Cell. Biol.,
5:3545, 1985; Hutchison et al, Proc.Natl.Acad.Sci. USA,
83:710, 1986; Derbyshire et al, Gene, 46:145, 1986; Zakour
et al, Nature, 295:708, 1982; Lehtovaara et al, Protein
Eng., 2:63, 1988; Leung et al, Technique, 1:11, 1989; Zhou
et al, Nucl. Acids Res., 19:6052, 1991).

The gene product can be selected, for example, by its
ability to bind a ligand or to carry out a chemical
reaction. (See, e.g., Joyce, Id.,1989; Robertson et al,
Nature, 344:467, 1990; Tuerk et al, Science, 249:505,
1990). The gene that corresponds to the selected gene
product can be amplified by a reciprocal primer method, such

as the polymerase chain reaction (PCR). (See Saiki et al,
Science, 230:1350-54, 1985; Saiki et al, Science, 239:487-491, 1988).

Alternatively, nucleic acid amplification may be
carried out using self-sustained sequence replication (3SR).
(See Guatelli et al, Proc.Natl.Acad.Sci USA, 87:1874, 1990,
the disclosures of which are incorporated by reference
herein.) According to the 3SR method, target nucleic acid
sequences may be amplified (replicated) exponentially *in
vitro* under isothermal conditions by using three enzymatic
activities essential to retroviral replication: (1) reverse
transcriptase, (2) RNase H, and (3) a DNA-dependent RNA
polymerase. By mimicking the retroviral strategy of RNA
replication by means of cDNA intermediates, this reaction
accumulates cDNA and RNA copies of the original target.

In summary, if one is contemplating the evolution of a
population of enzymatic DNA molecules, a continuous series
of reverse transcription and transcription reactions
replicates an RNA target sequence by means of cDNA
intermediates. The crucial elements of this design are (a)
the oligonucleotide primers both specify the target and
contain 5' extensions encoding the T7 RNA polymerase binding
site, so that the resultant cDNAs are competent
transcription templates; (b) cDNA synthesis can proceed to
completion of both strands due to the degradation of
template RNA in the intermediate RNA-DNA hybrid by RNase H;
and (c) the reaction products (cDNA and RNA) can function as
templates for subsequent steps, enabling exponential
replication.

If one is evolving enzymatic DNA molecules, various
critical elements of this design are somewhat different, as
disclosed in these Examples. For instance, (1) the
oligonucleotide primers specify the target and are

preferably "marked" or labeled in some fashion -- e.g., via
biotinylation -- so the resultant competent template strands
are easily identified; and (2) the *in vitro* selection
procedure used preferably depends upon the identification of

5   the most favorable release mechanism.

A major obstacle to realizing Darwinian evolution *in
vitro* is the need to integrate mutation and amplification,
both of which are genotype-related, with selection, which is
phenotype-related.   In the case of nucleic acid enzymes, for

10   which genotype and phenotype are embodied in the same
molecule, the task is simplified.

A.   Design of Enzymatic DNA Molecules

It is well known that single-stranded DNA can

15   assume interesting tertiary structures.   The structure of a
"tDNA", for example, closely resembles that of the
corresponding tRNA.   (See Paquette et al, Eur. J. Biochem.,
189:259-265, 1990).   Furthermore, it has been possible to
replace as many as 31 of 35 ribonucleotides within a

20   hammerhead ribozyme, while retaining at least some catalytic
activity.   (See Perreault et al, Nature, 344:565-567, 1990;
Williams et al, Proc. Natl. Acad. Sci. USA, 89:918-921,
1992;   Yang et al, Biochemistry, 31:5005-5009, 1992).

In vitro selection techniques have been applied to

25   large populations of random-sequence DNAs, leading to the
recovery of specific DNA "aptamers" that bind a target
ligand with high affinity (Bock et al, Nature, 355:564-566,
1992);   Ellington et al Nature, 355:850-852, 1992;   Wyatt et
al, Proc.Natl.Acad.Sci. USA, 91:1356-1360, 1994).   Recently,

30   two groups carried out the first NMR structural
determination of an aptamer, a 15mer DNA that forms a
G-quartet structure and binds the protein thrombin with high
affinity (Wang et al, Biochemistry, 32:1899-1904, 1993;

Macaya et al, _Proc.Natl.Acad.Sci. USA_, 90:3745-3749, 1993).
These findings were corroborated by an X-ray
crystallographic analysis (Padmanabhan et al, _J. Biol._
Chem., 268:17651-17654, 1993).

5   The ability to bind a substrate molecule with high
affinity and specificity is a prerequisite of a good enzyme.
In addition, an enzyme must make use of well-positioned
functional groups, either within itself or a cofactor, to
promote a particular chemical transformation. Furthermore,
10   the enzyme must remain unchanged over the course of the
reaction and be capable of operating with catalytic
turnover. Some would add the requirement that it be an
informational macromolecule, comprised of subunits whose
specific ordering is responsible for catalytic activity.
15   While these criteria are open to debate on both semantic and
chemical grounds, they serve to distinguish phenomena of
chemical rate enhancement that range from simple solvent
effects to biological enzymes operating at the limit of
substrate diffusion (Albery et al, _Biochemistry_,
20   15:5631-5640, 1976).

As described in greater detail hereinbelow, we sought
to develop a general method for rapidly obtaining DNA
catalysts and DNA enzymes, starting from random sequences.
As an initial target, we chose a reaction that we felt was
25   well within the capability of DNA: the hydrolytic cleavage
of an RNA phosphodiester, assisted by a divalent metal
cofactor. This is the same reaction that is carried out by
a variety of naturally-occurring RNA enzymes, including the
hammerhead and hairpin motifs. (See, e.g., Forster et al,
30   _Cell_, 49:211-220, 1987; Uhlenbeck, _Nature_, 328:596-600,
1987; Hampel et al, _Biochemistry_, 28:4929-4933, 1989).

It has recently been shown that, beginning with a
randomized library of tRNA molecules, one can obtain

ribozymes that have Pb^{2+}-dependent, site-specific RNA
phosphoesterase activity at neutral pH (Pan et al,
Biochemistry, 31:3887-3895, 1992; Pan et al, Nature,
358:560-563, 1992). This is analogous to the fortuitous

5   self-cleavage reaction of yeast $tRNA^{Phe}$ (Dirheimer et al,
Biochimie, 54:127-144, 1972), which depends on specific
coordination of a Pb^{2+} ion at a defined site within the
tRNA.   (See Rubin et al, J. Biomol. Struct. Dyn., 1:639-646,
1983; Brown et al, Biochemistry, 24:4785-4801, 1985).

10   As disclosed herein, our goals included the development
of DNAs that could carry out Pb^{2+}-dependent cleavage of a
particular RNA phosphoester, initially presented within a
short leader sequence attached to the 5' end of the DNA, and
ultimately located within a separate molecule that could be

15   cleaved in an intermolecular fashion with rapid catalytic
turnover. These goals were successfully achieved, as
described further below.

No assumptions were made as to how the DNA would
interact with the target phosphoester and surrounding

20   nucleotides. Beginning with a pool of approximately 10^{14}
random 50mer sequences, in vitro selection was allowed to
run its course. After five rounds of selection carried out
over four days, the population as a whole had attained the
ability to cleave the target phosphoester in the presence of

25   1 mM Pb^{2+} at a rate of about 0.2 min^{-1}. This is an
approximately 10^5-fold increase compared to the spontaneous
rate of cleavage under the same reaction conditions.

Individuals were isolated from the population,
sequenced, and assayed for catalytic activity. Based on

30   this information, the reaction was converted to an
intermolecular format and then simplified to allow
site-specific cleavage of a 19mer substrate by a 38mer DNA
enzyme, in a reaction that proceeds with a turnover rate of

1 min⁻¹ at 23°C and pH 7.0 in the presence of 1 mM PbOAc.

       B.   *In Vitro* Selection Scheme

        A starting pool of approximately 10^{14} single-
stranded DNA molecules was generated, all of which contain a
5' biotin moiety, followed successively by a fixed domain
that includes a single ribonucleotide, a potential catalytic
domain comprised of 50 random deoxyribonucleotides, and a
second fixed domain that lay at the 3' terminus (Figure 1).

        The pool was constructed by a nested PCR (polymerase
chain reaction) technique, beginning with synthetic DNA that
contained 50 random nucleotides flanked by primer binding
sites. The nested PCR primer was a 5'-biotinylated
synthetic oligodeoxynucleotide with a 3'-terminal adenosine
ribonucleotide. Ribonucleotide-terminated oligonucleotides
efficiently prime template-directed elongation in the
context of the PCR, in this case giving rise to an extension
product that contains a single embedded ribonucleotide.

        Figure 1 illustrates a selective amplification scheme
for isolation of DNAs that cleave a target RNA phosphoester.
Double-stranded DNA containing a stretch of 50 random
nucleotides is amplified via PCR, employing a
5'-biotinylated DNA primer (e.g., primer 3 -- 3a or 3b)
terminated at the 3' end by an adenosine ribonucleotide
(represented by the symbol "N" or "rA", wherein both N and
rA represent an adenosine ribonucleotide). This primer is
extended by *Taq* polymerase to yield a DNA product that
contains a single embedded ribonucleotide. The resulting
double-stranded DNA is immobilized on a streptavidin matrix
and the unbiotinylated DNA strand is removed by washing with
0.2 N NaOH. After re-equilibrating the column with a
buffered solution, the column is washed with the same
solution with added 1 mM PbOAc. DNAs that undergo

Pb^{2+}-dependent self-cleavage are released from the column,
collected in the eluant, and amplified by PCR. The PCR
products are then used to initiate the next round of
selective amplification.

5   The PCR products were passed over a streptavidin
affinity matrix, resulting in noncovalent attachment of the
5'-biotinylated strand of the duplex DNA. The
nonbiotinylated strand was removed by brief washing with 0.2
N NaOH, and the bound strand was equilibrated in a buffer
10   containing 0.5 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$, and 50 mM
HEPES (pH 7.0) at 23°C. Next, 1 mM PbOAc was provided in
the same buffer, allowing Pb^{2+}-dependent cleavage to occur
at the target phosphoester, thereby releasing a subset of
the DNAs from the streptavidin matrix. In principle, an
15   individual DNA might facilitate its own release by various
means, such as disruption of the interaction between biotin
and streptavidin or cleavage of one of the
deoxyribonucleotide linkages. It was felt that cleavage of
the ribonucleoside 3´-O-P bond would be the most likely
20   mechanism for release, based on the relative lability of
this linkage, and that Pb^{2+}-dependent hydrolytic cleavage
would allow release to occur most rapidly. In principle,
however, the *in vitro* selection procedure should identify
the most favorable release mechanism as well as those
25   individuals best able to carry out that mechanism.

         DNA molecules released from the matrix upon addition of
Pb^{2+} were collected in the eluant, concentrated by
precipitation with ethanol, and subjected to nested PCR
amplification. As in the construction of the starting pool
30   of molecules, the first PCR amplification utilized primers
that flank the random region (primers 1 and 2) and the
second utilized a 5'-biotinylated primer (primer 3b) that
has a 3´-terminal riboadenylate, thereby reintroducing the

target RNA phosphoester. The entire selective amplification procedure requires 3-4 hours to perform.

The molecules are purified in three ways during each round of this procedure: first, following PCR amplification, by extracting twice with phenol and once with chloroform / isoamyl alcohol, then precipitating with ethanol; second, following attachment of the DNA to streptavidin, by washing away all the nonbiotinylated molecules under strongly denaturing conditions; and third, following elution with Pb^{2+}, by precipitating with ethanol. There is no gel electrophoresis purification step, and thus no selection pressure constraining the molecules to a particular length.

C. Selection of Catalytic DNA

Five successive rounds of *in vitro* selection were carried out, progressively decreasing the reaction time following addition of Pb^{2+} in order to progressively increase the stringency of selection. During rounds 1 though 3, the reaction time was 1 hour; during round 4, the reaction time was 20 minutes; and during round 5, it was 1 minute. The starting pool of single-stranded DNAs, together with the population of molecules obtained after each round of selection, was assayed for self-cleavage activity under conditions identical to those employed during *in vitro* selection (see Figure 2).

For this assay, the molecules were prepared with a $5'-^{32}P$ rather than a 5'-biotin moiety, allowing detection of both the starting material and the 5' cleavage product. Following a 5-minute incubation, there was no detectable activity in the initial pool (G0) or in the population obtained after the first and second rounds of selection. DNAs obtained after the third round (G3) exhibited a modest level of activity; this activity increased steadily,

reaching approximately 50% self-cleavage for the DNAs
obtained after the fifth round of selection (G5). Cleavage
was detected only at the target phosphoester, even after
long incubation times. This activity was lost if Pb^{2+} was
5   omitted from the reaction mixture.

Figure 2 illustrates the self-cleavage activity of the
starting pool of DNA (G0) and populations obtained after the
first through fifth rounds of selection (G1 - G5). Reaction
mixtures contained 50 mM $MgCl_2$, 0.5 M NaCl, 0.5 M KCl, 50 mM
10   HEPES (pH 7.0 at 23°C), and 3 nM $[5'-^{32}P]$-labeled DNA,
incubated at 23°C for 5 min either in the presence or in the
absence of 1 mM PbOAc. The symbol **Pre** represents
108-nucleotide precursor DNA (SEQ ID NO 4); **Clv**,
28-nucleotide 5'-cleavage product (SEQ ID NO 5); and **M**,
15   primer 3a (SEQ ID NO 6), corresponding in length to the
5'-cleavage product.

The 28-nucleotide 5' cleavage product (Clv) illustrated
preferably has the sequence 5'-GGGACGAATTCTAATACGACTCACTATN-
3', wherein "N" represents adenosine ribonucleotide with an
20   additional 2', 3'-cyclic phosphate on the 3' end (SEQ ID NO
5). In alternative embodiments, "N" represents adenosine
ribonucleotide with an additional 2' or 3' phosphate on the
3' end of the molecule.

In Figure 2, the "G0" lane "Pre" band comprises a
25   sampling of 108-nucleotide precursor DNAs that each include
50 random nucleotides. Therefore, any given "Pre" sampling
will contain a wide variety of precursor DNAs, and each
sampling will likely differ from previous and subsequent
samplings. The "G1" through "G5" lanes contain "Pre" bands
30   that are increasingly enriched for catalytic DNA molecules,
but still contain a large number of different DNA sequences
(i.e., differing in the 50 nucleotide randomized domain). A
sample of these different sequences from "G5 Pre" DNA is

provided in Figure 3.

Shotgun cloning techniques were employed to isolate
individuals from the G5 population; the complete nucleotide
sequences of 20 of these subclones were then determined (see

5   Figure 3).   (Also see Cadwell et al, PCR Methods and
Applications, 2:28-33, 1992);   and Cadwell et al, PCR
Methods and Applications, 3(Suppl.):S136-S140, 1994).   Of
the 20 sequences, five were unique, two occurred twice, one
occurred three times, and one occurred eight times.   All of

10   the individual variants share common sequence elements
within the 50-nucleotide region that had been randomized in
the starting pool of DNA.   They all contain two presumed
template regions, one with complementarity to a stretch of
nucleotides that lies just upstream from the cleavage site

15   and the other with complementarity to nucleotides that lie
at least four nucleotides downstream.   Between these two
presumed template regions lies a variable domain of 1-11
nucleotides, followed by the fixed sequence 5'-AGCG-3', then
a second variable domain of 3-8 nucleotides, and finally the

20   fixed sequence 5'-CG-3' or 5'-CGA-3'.   Nucleotides that lie
outside of the two presumed template regions are highly
variable in both sequence and length.   In all of the
sequenced subclones, the region corresponding to the 50
initially-randomized nucleotides remains a total of 50

25   nucleotides in length.

Figure 3 illustrates the sequence alignment of
individual variants isolated from the population after five
rounds of selection.   The fixed substrate domain (5'-
GGGACGAATTCTAATACGACTCACTATrAGGAAGAGATGGCGAC-3', or 5'-

30   GGGACGAATTCTAATACGACTCACTATNGGAAGAGATGGCGAC-3', where N
represents adenosine ribonucleotide) (SEQ ID NO 13) is shown
at the top, with the target riboadenylate identified with an
inverted triangle.   Substrate nucleotides that are commonly

involved in presumed base-pairing interactions are indicated
by a vertical bar. Sequences corresponding to the 50
initially-randomized nucleotides are aligned antiparallel to
the substrate domain. All of the variants are 3'-terminated

5   by the fixed sequence 5'-CGGTAAGCTTGGCAC-3' (SEQ ID NO 1)
("primer site"; not shown). Nucleotides within the
initially-randomized region that are presumed to form base
pairs with the substrate domain are indicated on the right
and left sides of the Figure; the putative base-pair-forming

10   (or substrate binding) regions of the enzymatic DNA
molecules are individually boxed in each sequence shown.
The highly-conserved nucleotides within the putative
catalytic domain are illustrated in the two boxed columns.

        While it is anticipated that additional data will be

15   helpful in constructing a meaningful secondary structural
model of the catalytic domain, we note that, like the
hammerhead and hairpin ribozymes, the catalytic domain of
our enzymatic DNA molecules appears to contain a conserved
core flanked by two substrate binding regions (or

20   recognition domains) that interact with the substrate
through base-pairing interactions. Similar to the
hammerhead and hairpin ribozymes, the catalytic DNAs also
appear to require a short stretch of unpaired substrate
nucleotides -- in this case 5'-GGA-3' -- between the two

25   regions that are involved in base pairing.

        It was also interesting to note that each of the nine
distinct variants exhibited a different pattern of presumed
complementarity with the substrate domain. In some cases,
base pairing was contiguous, while in others it was

30   interrupted by one or more noncomplementary pairs. The
general tendency seems to be to form tighter interaction
with the nucleotides that lie upstream from the cleavage
site compared to those that lie downstream. Binding studies

●   ●

and site-directed mutagenesis analysis should enable us to gain further insights and to further substantiate this conjecture.

In order to gain further insight into the sequence requirements for catalytic function, the self-cleavage activity of six of the nine variants was tested and evaluated under the within-described selection conditions (see Figure 3). Not surprisingly, the sequence that occurred in eight of the 20 subclones proved to be the most reactive, with a first-order rate constant of 1.4 min^{-1}. All of the studied variants were active in the self-cleavage assay and all gave rise to a single 5'-labeled product corresponding to cleavage at the target RNA phosphoester.

The dominant subclone was further analyzed under a variety of reaction conditions. Its self-cleavage activity was dependent on Pb^{2+} but was unaffected if Mg^{2+} was omitted from the reaction mixture. There was a requirement for a monovalent cation as well, which can be met by either Na^+ or K^+. The reaction rate increased linearly with increasing concentration of monovalent cation over the range of 0 - 1.0 M (r = 0.998). Other variables that may affect the reaction, such as pH, temperature, and the presence of other divalent metals, are in the process of being evaluated further.

2.   Materials and Methods

    A.   Oligonucleotides and Oligonucleotide Analogs

        Synthetic DNAs and DNA analogs were purchased from Operon Technologies. The 19-nucleotide substrate, 5'-pTCACTATrAGGAAGAGATGG-3' (or 5'-pTCACTATNGGAAGAGATGG-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 7), was prepared by reverse-transcriptase catalyzed extension of 5'-pTCACTATrA-3' (or 5'-pTCACTATN-3', wherein

"N" represents adenosine ribonucleotide) (SEQ ID NO 8), as
previously described (Breaker et al, <u>Biochemistry</u>,
33:11980-11986, 1994), using the template
5'-CCATCTCTTCCTATAGTGAGTCCGGCTGCA-3' (SEQ ID NO 9).   Primer
3, 5'-GGGACGAATTCTAATACGACTCACTATrA-3' (or 5'-
GGGACGAATTCTAATACGACTCACTATN-3', wherein "N" represents
adenosine ribonucleotide) (SEQ ID NO 6), was either
5'-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase
(primer 3a) or 5'-thiophosphorylated with [γ-S]ATP and T4
polynucleotide kinase and subsequently biotinylated with
N-iodoacetyl-N'-biotinylhexylenediamine (primer 3b).

B.   DNA Pool Preparation
       The starting pool of DNA was prepared by PCR using
the synthetic oligomer
5'-GTGCCAAGCTTACCG-N$_{50}$-GTCGCCATCTCTTCC-3' (SEQ ID NO 4),
where N is an equimolar mixture of G, A, T and C.   A 2-ml
PCR, containing 500 pmoles of the randomized oligomer, 1,000
pmoles primer 1 (5'-GTGCCAAGCTTACCG-3', SEQ ID NO 10), 500
pmoles primer 2
(5'-CTGCAGAATTCTAATACGACTCACTATAGGAAGAGATGGCGAC-3', SEQ ID
NO 11), 500 pmoles primer 3b, 10 μCi [α-^{32}P]dATP, and 0.2 U
μl^{-1} Taq DNA polymerase, was incubated in the presence of 50
mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl (pH 8.3 at 23°C), 0.01%
gelatin, and 0.2 mM of each dNTP for 1 min at 92°C, 1 min at
50°C, and 2 min at 72°C, then 5 cycles of 1 min at 92°C, 1
min at 50°C, and 1 min at 72°C.   The resulting mixture was
extracted twice with phenol and once with chloroform /
isoamyl alcohol, and the DNA was isolated by precipitation
with ethanol.

C.   <i>In Vitro</i> Selection
       The starting pool of DNA was resuspended in 500 μL

of buffer A (1 M NaCl and 50 mM HEPES (pH 7.0 at 23°C)) and
was passed repeatedly over a streptavidin column (AffiniTip
Strep 20, Genosys, The Woodlands, TX). The column was
washed with five 100-μl volumes of buffer A, followed by

5   five 100-μl volumes of 0.2 N NaOH, then equilibrated with
five 100-μl volumes of buffer B (0.5 M NaCl, 0.5 M KCl, 50
mM MgCl$_2$, and 50 mM HEPES (pH 7.0 at 23°C)). The
immobilized single-stranded DNA was eluted over the course
of 1 hr with three 20-μl volumes of buffer B with added 1 mM

10   PbOAc. The entire immobilization and elution process was
conducted at 23°C. The eluant was collected in an equal
volume of buffer C (50 mM HEPES (pH 7.0 at 23°C) and 80 mM
EDTA) and the DNA was precipitated with ethanol.

        The resulting DNA was amplified in a 100-μL PCR

15   containing 20 pmoles primer 1, 20 pmoles primer 2, 0.05 U
μl^{-1} Taq polymerase, 50 mM KCl, 1.5 mM MgCl$_2$, 10 mM Tris-HCl
(pH 8.3 at 23°C), 0.01% gelatin, and 0.2 mM of each dNTP for
30 cycles of 10 sec at 92°C, 30 sec at 50°C, and 30 sec at
72°C. The reaction products were extracted twice with

20   phenol and once with chloroform / isoamyl alcohol, and the
DNA was recovered by precipitation with ethanol.
Approximately 4 pmoles of the amplified DNA was added to a
second, nested PCR containing 100 pmoles primer 1, 100
pmoles primer 3b, 20 μCi [α-^{32}P]dATP, and 0.1 U μl^{-1} Taq

25   polymerase, in a total volume of 200 μL that was amplified
for 10 cycles of 1 min at 92°C, 1 min at 50°C, and 1 min at
72°C. The PCR products were once more extracted and
precipitated, and the resulting DNA was resuspended in 50 μL
buffer A, then used to begin the next round of selection.

30   The second and third rounds were carried out as above,
except that the nested PCR at the end of the third round was
performed in a 100-μl volume. During the fourth round, the
elution time following addition of Pb^{2+} was reduced to 20

min (two 20-μL elution volumes) and only half of the
recovered DNA was used in the first PCR, which involved only
15 temperature cycles. During the fifth round, the elution
time was reduced to 1 min (two 20-μL elution volumes) and
only one-fourth of the recovered DNA was used in the first
PCR, which involved 15 temperature cycles. DNA obtained
after the fifth round of selection was subcloned and
sequenced, as described previously (Tsang et al,
Biochemistry, 33:5966-5973, 1994).

D.   Kinetic Analysis of Catalytic DNAs

Populations of DNA and various subcloned
individuals were prepared with a 5'-^{32}P label by asymmetric
PCR in a 25-μl reaction mixture containing 10 pmoles primer
3a, 0.5 pmoles input DNA, and 0.1 U μl^{-1} Taq polymerase,
under conditions as described above, for 10 cycles of 1 min
at 92°C, 1 min at 50°C, and 1 min at 72°C. The resulting
[5'-^{32}P]-labeled amplification products were purified by
electrophoresis in a 10% polyacrylamide / 8 M gel.

Self-cleavage assays were carried out following
preincubation of the DNA in buffer B for 10 min. Reactions
were initiated by addition of PbOAc to 1 mM final
concentration and were terminated by addition of an equal
volume of buffer C. Reaction products were separated by
electrophoresis in a 10% polyacrylamide / 8 M gel. Kinetic
assays under multiple-turnover conditions were carried out
in buffer B that included 50 μg ml^{-1} BSA to prevent
adherence of material to the vessel walls. Substrate and
enzyme molecules were preincubated separately for 5 min in
reaction buffer that lacked Pb^{2+}, then combined, and the
reaction was initiated by addition of PbOAc to a final
concentration of 1 mM.

3.   Evolution of Deoxyribozymes That Cleave
      Intermolecularly

   A.   Conversion to an Intermolecular Format

      Based on the variable pattern of presumed

5 base-pairing interactions between the catalytic and

   substrate domains of the studied variants, it was felt that

   it would be reasonably straightforward to convert the

   DNA-catalyzed reaction to an intermolecular format. In

   doing so, we wished to simplify the two substrate-binding

10 regions of the catalyst so that each would form an

   uninterrupted stretch of 7-8 base pairs with the substrate.

   In addition, we wished to provide a minimal substrate,

   limited to the two base-pairing regions and the intervening

   sequence 5'-GGA-3' (Figure 4A).

15   Figures 4A and 4B illustrate DNA-catalyzed cleavage of

   an RNA phosphoester in an intermolecular reaction that

   proceeds with catalytic turnover. Figure 4A is a

   diagrammatic representation of the complex formed between

   the 19mer substrate and 38mer DNA enzyme. The substrate

20 contains a single adenosine ribonucleotide ("rA" or "N",

   adjacent to the arrow), flanked by deoxyribonucleotides.

   The synthetic DNA enzyme is a 38-nucleotide portion of the

   most frequently occurring variant shown in Figure 3.

   Highly-conserved nucleotides located within the putative

25 catalytic domain are "boxed". As illustrated, one conserved

   sequence is "AGCG", while another is "CG" (reading in the

   5'-3' direction).

      Figure 4B shows an Eadie-Hofstee plot used to determine

   K_m (negative slope) and V_{max} (y-intercept) for DNA-catalyzed

30 cleavage of [5'-^{32}P]-labeled substrate under conditions

   identical to those employed during *in vitro* selection.

   Initial rates of cleavage were determined for reactions

   involving 5 nM DNA enzyme and either 0.125, 0.5, 1, 2, or 4

μM substrate.

In designing the catalytic domain, we relied heavily on the composition of the most reactive variant, truncating by two nucleotides at the 5' end and 11 nucleotides at the 3' end. The 15 nucleotides that lay between the two template regions were left unchanged and a single nucleotide was inserted into the 3' template region to form a continuous stretch of nucleotides capable of forming base pairs with the substrate. The substrate was simplified to the sequence 5'-<u>TCACTAT</u>rA ● GG<u>AAGAGA</u>TGG-3' (or 5'-<u>TCACTAT</u>N ● GG<u>AAGAGA</u>TGG-3', wherein "N" represents adenosine ribonucleotide) (SEQ ID NO 12), where the underlined nucleotides correspond to the two regions involved in base pairing with the catalytic DNA molecule.

The simplified reaction system, employing a 38mer catalytic DNA molecule (catalyst) comprised entirely of deoxyribonucleotides and a 19mer substrate containing a single ribonucleotide embedded within an otherwise all-DNA sequence, allows efficient DNA-catalyzed phosphoester cleavage with rapid turnover. Over a 90-minute incubation in the presence of 0.01 μM catalyst and 1 μM substrate, 46% of the substrate is cleaved, corresponding to 46 turnovers of the catalyst. A preliminary kinetic analysis of this reaction was carried out, evaluated under multiple-turnover conditions. The DNA catalyst exhibits Michaelis-Menten kinetics, with values for k_{cat} and K_m of 1 min^{-1} and 2 μM, respectively (see Figure 4B). The value for K_m is considerably greater than the expected dissociation constant between catalyst and substrate based on Watson-Crick interactions. The substrate was incubated under identical reaction conditions (but in the absence of the catalyst); a value for k_{uncat} of 4 $\times$ 10^{-6} min^{-1} was obtained. This is consistent with the reported value of 5 x 10^{-3} min^{-1} for

hydrolysis of the more labile 1-nitrophenyl-1,2-propanediol in the presence of 0.5 mM Pb^{2+} at pH 7.0 and 37°C (Breslow et al, Proc.Natl.Acad.Sci. USA, 88:4080-4083, 1991).

It is now presumed that the phosphoester cleavage reaction proceeds via a hydrolytic mechanism involving attack by the ribonucleoside 2′-hydroxyl on the vicinal phosphate, generating a 5' product with a terminal 2'(3')-cyclic phosphate and 3' product with a terminal 5'-hydroxyl. In support of this mechanism, the 3'-cleavage product is efficiently phosphorylated with T4 polynucleotide kinase and $[\gamma-^{32}P]$ATP, consistent with the availability of a free 5'-hydroxyl.

B.   Discussion

After five rounds of *in vitro* selection, a population of single-stranded DNA molecules that catalyze efficient Pb^{2+}-dependent cleavage of a target RNA phosphoester was obtained. Based on the common features of representative individuals isolated from this population, a simplified version of both the catalytic and substrate domains was constructed, leading to a demonstration of rapid catalytic turnover in an intermolecular context. Thus the 38mer catalytic domain provides an example of a DNA enzyme, or what might be termed a "deoxyribozyme".

Referring to this molecule as an enzyme, based on the fact that it is an informational macromolecule capable of accelerating a chemical transformation in a reaction that proceeds with rapid turnover and obeys Michaelis-Menten kinetics, may not satisfy everyone's notion of what constitutes an enzyme. Some might insist that an enzyme, by definition, must be a polypeptide. If, however, one accepts the notion of an RNA enzyme, then it seems reasonable to adopt a similar view concerning DNA enzymes. Considering

how quickly we were able to generate this molecule from a
pool of random-sequence DNAs, we expect that many other
examples of synthetic DNA enzymes will appear in the near
future.

5   The Pb^{2+}-dependent cleavage of an RNA phosphoester was
chosen as an initial target for DNA catalysis because it is
a straightforward reaction that simply requires the proper
positioning of a coordinated Pb^{2+}-hydroxyl to facilitate
deprotonation of the 2´ hydroxyl that lies adjacent to the

10   cleavage site. (See Pan et al, in The RNA World, Gesteland
& Atkins (eds.), pp. 271-302, Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, NY (1993).) Pb^{2+} is known to
coordinate to the N7 position of purines, the O6 position of
guanine, the O4 position of uracil, and the N3 position of

15   cytosine (Brown et al, Nature, 303:543-546, 1993). Thus,
the differences in sugar composition and conformation of DNA
compared to RNA seemed unlikely to prevent DNA from forming
a well-defined Pb^{2+}-binding pocket.

           A substrate that contains a single ribonucleotide

20   within an otherwise all-DNA sequence was chosen because it
provided a uniquely favored site for cleavage and insured
that any resulting catalytic activity would be attributable
solely to DNA. Substrate recognition appears to depend on
two regions of base-pairing interactions between the

25   catalyst and substrate. However, the unpaired substrate
nucleotides, 5'-GGA-3', that lie between these two regions
may play an important role in substrate recognition, metal
coordination, or other aspects of catalytic function.

           It is further anticipated that an all-RNA molecule,

30   other RNA-DNA composites, and molecules containing one or
more nucleotide analogs may be acceptable substrates. As
disclosed herein, the within-described in vitro evolution
procedures may successfully be used to generate enzymatic

DNA molecules having the desired specificities; further
analyses along these lines are presently underway.

In addition, studies to determine whether the presumed
base-pairing interactions between enzyme and substrate are
5   generalizable with respect to sequence are in progress,
using the presently-described methods. The within-disclosed
Pb^{2+}-dependent deoxyribozymes may also be considered model
compounds for exploring the structural and enzymatic
properties of DNA.

10   The methods employed in the present disclosure for the
rapid development of DNA catalysts will have considerable
generality, allowing us to utilize other cofactors to
trigger the cleavage of a target linkage attached to a
potential catalytic domain. In this regard, the development

15   of Mg^{2+}-dependent DNA enzymes that specifically cleave
target RNAs under physiological conditions is of interest,
as is the development of DNA enzymes that function in the
presence of other cations (see Example 4). Such molecules
will provide an alternative to traditional antisense and

20   ribozyme approaches for the specific inactivation of target
mRNAs.

DNA thus joins RNA and protein on the list of
biological macromolecules that are capable of exhibiting
enzymatic activity. The full extent of DNA's catalytic

25   abilities remains to be explored, but these explorations
should proceed rapidly based on *in vitro* selection methods
such as those employed in this study.

DNA enzymes offer several important advantages compared
to other macromolecular catalysts. First, they are easy to

30   prepare, in an era when most laboratories have access to an
automated DNA synthesizer and the cost of DNA
phosphoramidites has become quite modest. Second, they are
very stable compounds, especially compared to RNA, thus

facilitating their use in biophysical studies. Third, we
expect that they can be adapted to therapeutic applications
that at present make use of antisense DNAs that lack
RNA-cleavage activity. *In vitro* selection could be carried

5   out with DNA analogs, including compounds that are nuclease
resistant such as phosphorothioate-containing DNA, so long
as these analogs can be prepared in the form of a
deoxynucleoside 5'-triphosphate and are accepted as a
substrate by a DNA-dependent DNA polymerase. Finally, DNA

10   enzymes offer a new window on our understanding of the
macromolecular basis of catalytic function. It will be
interesting, for example, to carry out comparative analyses
of protein-, RNA-, and DNA-based enzymes that catalyze the
same chemical transformation.

15

4.   Other Families of Catalytic DNAs

A starting pool of DNA was prepared by PCR essentially
as described in Example 2.B. above, except that the starting
pool of DNA comprised molecules containing 40 random

20   nucleotides. Thus, the starting pool of DNA described
herein was prepared by PCR using the synthetic oligomer 5´
GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA
CAT CTC N_{40}GT GAC GGT AAG CTT GGC AC 3´ (SEQ ID NO 23),
where N is an equimolar mixture of G, A, T and C, and where

25   the DNA molecules were selected for the ability to cleave
the phosphoester following the target rA. (See Figure 6A,
also.)

Selective amplification was carried out in the presence
of either Pb^{2+}, Zn^{2+}, Mn^{2+}, or Mg^{2+}, thereby generating at least

30   four "families" of catalytic DNA molecules. As illustrated
in Figure 5, catalytic DNA molecules demonstrating specific
activity were generated in the presence of a variety of
cations.

Figure 5 is a photographic representation showing a polyacrylamide gel demonstrating specific endoribonuclease activity of four families of selected catalytic DNAs. Selection of a Pb^{2+}-dependent family of molecules was

5   repeated in a side-by-side fashion as a control. In each group of three lanes, the first lane shows the lack of activity of the selected population in the absence of the metal cation, the second lane shows the observed activity in the presence of the metal cation, and the third lane shows

10   the lack of activity of the starting pool (G0). At present, the order of reactivity is observed to be $Pb^{2+}>Zn^{2+}>Mn^{2+}>Mg^{2+}$, mirroring the pK_a of the corresponding metal-hydroxide.

After either five (G5) or six (G6) rounds of selective amplification in the presence of the preselected divalent

15   cation, the desired endonuclease activity was obtained. The following description of selective amplification in the presence of Mg^{2+} is intended to be exemplary.

Six rounds of *in vitro* selective amplification were carried out, following the method described in Example 2

20   hereinabove, except that the divalent metal used was 1 mM Mg^{2+} rather than 1 mM Pb^{2+}. (See also Breaker et al, <u>Chem. & Biol.</u>, 1:223-229, 1994), incorporated by reference herein, which describes essentially the same procedure.)

Individual clones were isolated following the sixth

25   round, and the nucleotide sequence of 24 of these clones was determined. All of the sequences began with: 5´ GGG ACG AAT TCT AAT ACG ACT CAC TAT rA GG AAG AGA TGG CGA CA (SEQ ID NO 23 from position 1 to 44) and ended with: CGG TAA GCT TGG CAC   3´ (SEQ ID NO 23 from position 93 to 107).

30   The segment in the middle, corresponding to TCTC N_{40} GTGA (SEQ ID NO 23 from position 45 to 92) in the starting pool, varied as follows:

(13) **CCG** CCC ACC TCT TTT ACG AGC CTG TAC GAA ATA GTG
       CTC TTG TTA GTA **T** (SEQ ID NO 24)

(5)   TCT CTT CAG CGA TGC ACG CTT GTT TTA ATG TTG CAC
       CCA TGT TAG TGA (SEQ ID NO 25)

(2)   TCT CAT CAG CGA TTG AAC CAC TTG GTG GAC AGA CCC
       ATG TTA GTG A (SEQ ID NO 26)

(1)   **CCG** CCC ACC TCT TTT ACG AGC CTG TAC GAA ATA GTG
       TTC TTG TTA GTA **T** (SEQ ID NO 27)

(1)   **CCG** CCC ACC TCT TTT ACG AGC CTG TAC GAA ATA GTG
       CTC TCG TTA GTA **T** (SEQ ID NO 28)

(1)   TCT CAG ACT TAG TCC ATC ACA CTC TGT GCA TAT GCC
       TGC TTG ATG TGA (SEQ ID NO 29)

(1)   -CT CTC ATC TGC TAG CAC GCT CGA ATA GTG TCA GTC
       GAT GTG A (SEQ ID NO 30).

The initial number in parentheses indicates the number
of clones having that particular sequence. Note that some
mutations (highlighted in bold type) occurred at nucleotide
positions other than those that were randomized initially.

The second sequence listed above (i.e., SEQ ID NO 25),
which occurred in 5 of 24 clones, was chosen as a lead (i.e.
principal) compound for further study. Its cleavage
activity was measured in the presence of a 1 mM
concentration of various divalent metals and 1 M NaCl at pH
7.0 and 23°C:

metal	k_{obs} (min^{-1})
none	n.d.
Mg^{2+}	2.3×10^{-3}
Mn^{2+}	6.8×10^{-3}
Zn^{2+}	4.2×10^{-2}
Pb^{2+}	1.1×10^{-2}

Thus, the lead compound is active in the presence of all four divalent metals, even though it was selected for activity in the presence of Mg^{2+}. Conversely, DNA molecules that were selected for activity in the presence of Mn^{2+},

5   Zn^{2+}, or Pb^{2+} did not show any activity in the presence of Mg^{2+}.

In addition, the population of DNAs obtained after six rounds of *in vitro* selection in the presence of Mg^{2+}, when prepared as all-phosphorothioate-containing DNA analogs,

10   showed Mg^{2+}-dependent cleavage activity at an observed rate of $\sim 10^{-3}$ min^{-1}. The phosphorothioate-containing analogs were prepared enzymatically so as to have an R_p configuration at each stereocenter. Such compounds are relatively resistant to degradation by cellular nucleases compared to unmodified

15   DNA.

The lead compound was re-randomized at 40 nucleotide positions (underlined), introducing mutations at a frequency of 15% (5% probability of each of the three possible base substitutions). The re-randomized population was subjected

20   to seven additional rounds of *in vitro* selection. During the last four rounds, molecules that were reactive in the presence of 1 mM Pb^{2+} were removed from the population before the remainder were challenged to react in the presence of 1 mM Mg^{2+}. Individual clones were isolated

25   following the seventh round and the nucleotide sequence of 14 of these clones was determined. All of the sequences began with: 5´ GGG ACG AAT TCT AAT ACG ACT CAC TAT **rA** GG AAG AGA TGG CGA CAT CTC (SEQ ID NO 23, from position 1 to 48), and ended with: GTG ACG GTA AGC TTG GCA C 3´ (SEQ ID

30   NO 23, from position 89 to 107).

The segment in the middle, corresponding to the 40 partially-randomized positions (N_{40}, SEQ ID NO 23, from position 49 to 88), varied as follows:

(4) TAC AGC GAT **TCA** CCC TTG TTT **AAG GGT** TAC ACC CAT
     GTT A (SEQ ID NO 31)

(2) **ATC** AGC GAT **TAA** CGC TTG TTT **CAA TGT** TAC ACC CAT
     GTT A (SEQ ID NO 32)

5   (2) **TTC** AGC GAT **TAA** CGC TT**A** TTT **TAG CGT** TAC ACC CAT
     GTT A (SEQ ID NO 33)

(1) **ATC** AGC GAT **TCA** CCC TTG TTT **TAA GGT TGC** ACC CAT
     GTT A (SEQ ID NO 34)

(1) **ATC** AGC GAT **TCA** CCC TTG TTT **AAG CGT** TAC ACC CAT
10   GTT **G** (SEQ ID NO 35)

(1) **ATC** AGC GAT **TCA** CCC TTG TTT **TAA GGT** TAC ACC CAT
     GTT A (SEQ ID NO 36)

(1) **ATC** AGC GAT **TAA** CGC TT**A** TTT **TAG CGT** TAC ACC CAT
     GTT A (SEQ ID NO 37)

15   (1) **ATC** AGC GAT **TAA** CGC TTG TTT **TAG TGT TGC** ACC CAT
     GTT A (SEQ ID NO 38)

(1) **ATC** AGC GAT **TAA** CGC TT**A** TTT **TAG CAT** TAC ACC CAT
     GTT A (SEQ ID NO 39).

20   The number in parentheses indicates the number of
clones having that particular sequence. Nucleotides shown
in bold are those that differ compared to the lead compound.
     Formal analysis of the cleavage activity of these
clones is ongoing. The population as a whole exhibits
25   Mg^{2+}-dependent cleavage activity at an observed rate of $\sim10^{-2}$
min^{-1}, with a comparable level of activity in the presence
of Pb^{2+}.
     Figures 6A and 6B provide two-dimensional illustrations
of a "progenitor" catalytic DNA molecule and one of several
30   catalytic DNA molecules obtained via the selective
amplification methods disclosed herein, respectively.
Figure 6A illustrates an exemplary molecule from the
starting pool, showing the overall configuration of the

molecules represented by SEQ ID NO 23. As illustrated,
various complementary nucleotides flank the random (N_{40})
region.

Figure 6B is a diagrammatic representation of one of
the Mg^{2+}-dependent catalytic DNA molecules (or "DNAzymes")
generated via the within-described procedures. The location
of the ribonucleotide in the substrate nucleic acid is
indicated via the arrow.   (The illustrated molecule
includes the sequence identified herein as SEQ ID NO 25, as
well as "beginning" and "ending" sequences of SEQ ID NO 23.)

Endonuclease activity is continuing to be enhanced in
each of the aforementioned "families" via *in vitro*
evolution, as disclosed herein, so it is anticipated that
enzymatic DNA molecules of increasingly desirable
specificities may be generated successfully using the
within-disclosed guidelines.

5.   Cleavage of Larger RNA Sequences

As an extension of the foregoing, we have developed DNA
enzymes that cleave an all-RNA substrate, rather than a
single ribonucleotide embedded within an otherwise all-DNA
substrate as demonstrated above.   (Also see Breaker et al,
Chem. & Biol., 1:223-229, 1994); Breaker et al, Chem. &
Biol., 2:655-660, 1995). As a target sequence, we chose a
stretch of 12 highly-conserved nucleotides within the U5 LTR
region of HIV-1 RNA, having the sequence 5' GUAACUAGAGAU 3'
(SEQ ID NO 49).

Following the methods described in the previous
examples, we generated a pool of 10^{14} DNA molecules that
have the following composition:·

5'- GGAAAA **r(GUAACUAGAGAU)** GGAAGAGATGGCGAC **N_{50}**
         CGGTAAGCTTGGCAC   -3'   (SEQ ID NO 50),

where **N** is an equimolar mixture of the deoxyribonucleotides

G, A, T, and C, and where the sequence identified as
"r(GUAACUAGAGAU)" (SEQ ID NO 49) is comprised of *ribo*nucleotides.
(Optionally, one may alter the initial 5' nucleotide
sequence, e.g., by adding an additional dA residue to the
sequence preceding the ribonucleotide portion at the 5' end,
thus causing the initial sequence to read "GGAAAAA" and
causing SEQ ID NO 50 to be 99 residues in length. Clearly,
this is but one example of the modifications that may be
made in order to engineer specific enzymatic DNA molecules,
as disclosed in detail herein.)

       The initial library was generated by template-directed
extension of 50 pmols of
5'-biotin-d(GGAAAAA)r(GUAACUAGAGAU)d(GGAAGAGATGGCGAG)-3' (SEQ ID NO 4) on
100 pmols of 5'-GTGCCAAGCTTACCG-N50-GTCGCCATCTCTTCC-3' insert (N =
G, A, T or C), in a 50-ul reaction mixture containing 10 U
ul^{-1} Superscript II reverse transcriptase (RT; Gibco BRL), 3
mM MgCl2, 75 mM KCl, 50 mM Tris*HCl (pH 8.3), and 0.2 mM of
each dNTP. A trace amount of [5'-32P]-labeled primer was
included in the reaction mixture to allow extension
efficiency to be monitored. All components except RT were
combined, incubated at 65 C for 5 min, then cooled to 45 C
over 10 min. RT was added and the mixture was incubated at
45 C for 45 min, then quenched by addition of Na2 EDTA.
NaCl was added to a final concentration of 1 M and the
extension products were immobilized by repeated passing
through four streptavidin affinity columns (Genosys). The
columns were washed with five 100-ul volumes of wash buffer
(1 M NaCl, 50 mM Tris*HCl (pH 7.5), 0.1 mM Na2EDTA),
followed by five 100-ul volumes of 0.1 N NaOH and five
100-ul volumes of wash buffer at 37 C, then eluted at 37 C
over 1 hr with three 20-ul aliquots of reaction buffer (10
mM MgCl2, 1 M NaCl, 50 mM Tris*HCl (pH 7.5)). Eluted
molecules were recovered and amplified by the polymerase

chain reaction (PCR) using the primers
5'-biotin-GGAAGAGATGGCGAC-3' and 5'-GTGCCAAGCTTACCG-3'. The
PCR products were immobilized on streptavidin columns, as
above, which were washed with five 100-ul volumes of wash
buffer and eluted with 40 ul of 0.1 N NaOH to obtain the
non-biotinylated strand. The isolated DNAs were ethanol
precipitated and used as templates in a primer extension
reaction to begin the next round of selection. Rounds 2-10
were carried out as above, except that the reaction scale
was reduced five-fold during the extension step and two-fold
during PCR.

The enzymatic DNA molecules thus produced were selected
for their ability to cleave a phosphoester that lies within
the embedded RNA target sequence. Ten rounds of *in vitro*
selective amplification were carried out, based on the
enzymatic DNA molecules' activity in the presence of 10 mM
Mg^{2+} at pH 7.5 and 37°C. During the selection process,
there was competition for "preferred" cleavage sites as well
as for the "best" catalyst that cleaves at each such
preferred site. Two sites and two families of catalysts
emerged as possessing the most efficient cleavage
capabilities (see Figure 7).

Figure 7 illustrates some of the results of ten rounds
of *in vitro* selective amplification carried out essentially
as described herein. As shown, two sites and two families
of catalysts emerged as displaying the most efficient
cleavage of the target sequence. Cleavage conditions were
essentially as indicated in Figure 7, namely, 10mM Mg^{2+}, pH
7.5, and 37°; data collected after the reaction ran for 2
hours is shown. Cleavage (%) is shown plotted against the
number of generations (here, 0 through 10). The
number/prevalence of catalytic DNA molecules capable of
cleaving the target sequence at the indicated sites in the

substrate is illustrated via the vertical bars, with
cleavage at G↓UAACUAGAGAU (SEQ ID NO 49) shown by the striped bars, and
with cleavage at GUAACUA↓GAGAU (SEQ ID NO 49) illustrated via the open
(lightly-shaded) bars. In Figure 7, as herein, the arrow

5   (↓) indicates the site between two neighboring nucleotides
at which cleavage occurs.

Various individuals from the population obtained after
the 8th and 10th rounds of selective amplification were
cloned. The nucleotide sequences of 29 individuals from the

10   8th round and 32 individuals from the 10th round were then
determined (see Tables 2 and 3, respectively).

Under the heading "Nucleotide Sequence" in each of
Tables 2 and 3 is shown the portion of each identified clone
that corresponds to the 50 nucleotides that were randomized

15   in the starting pool (i.e., N_{50}); thus, the entire
nucleotide sequence of a given clone generally includes the
nucleotide sequences preceding, following, and including the
"N_{50}" segment, presuming the substrate sequence is attached
and that self-cleavage has not occurred. For example, the

20   entire sequence of a (non-self-cleaved) clone may generally
comprise residue nos. 1-33 of SEQ ID NO 50, followed by the
residues representing the randomized N_{50} region, followed by
residue nos. 84-98 of SEQ ID NO 50, or by residue nos. 1-34
of SEQ ID NO 51, followed by the residues representing the

25   randomized N_{50} region, followed by residue nos. 85-99 of SEQ
ID NO 51. It is believed, however, that the N_{50} (or N_{40})
region -- or a portion thereof -- of each clone is
particularly important in determining the specificity and/or
activity of a particular enzymatic DNA molecule. This is

30   particularly evident in reactions in which the substrate and
the DNAzyme are separate molecules (see, e.g., Figs. 8 and
9).

Clone numbers are designated as 8-x or 10-x for

individuals obtained after the 8th or 10th rounds, respectively. SEQ ID NOS are also listed and correspond to the "N_{50}" region of each clone.

Table 2

Cloned Individuals from 8th Round of Amplification

Clone No.	SEQ ID NO	"N_{50}" Nucleotide Sequence (5'→3')
8-2	52	CCA ATA GTG CTA CTG TGT ATC TCA ATG CTG GAA ACA CGG GTT ATC TCC CG
8-4	53	CCA AAA CAG TGG AGC ATT ATA TCT ACT CCA CAA AGA CCA CTT TTC TCC CG
8-5[1]	54	ATC CGT ACT AGC ATG CAG ACA GTC TGT CTG CTT TTT CAT TAC TCA CTC CC
8-14	55	CAA TTC ATG ATG ACC AAC TCT GTC AAC ACG CGA ACT TTT AAC ACT GGC A
8-17[2]	56	CTT CCA CCT TCC GAG CCG GAC GAA GTT ACT TTT TAT CAC ACT ACG TAT TG
8-3	57	GGC AAG AGA TGG CAT ATA TTC AGG TAA CTG TGG AGA TAC CCT GTC TGC CA
8-6	58	CTA GAC CAT TCA CGT TTA CCA AGC TAT GGT AAG AAC TAG AAT CAC GCG TA
8-8	59	CGT ACA CGT GGA AAA GCT ATA AGT CAA GTT CTC ATC ATG TAC CTG ACC GC
8-10	60	CAG TGA TAC ATG AGT GCA CCG CTA CGA CTA AGT CTG TAA CTT ATT CTA CC
8-22	61	ACC GAA TTA AAC TAC CGA ATA GTG TGG TTT CTA TGC TTC TTC TTC CCT GA
8-11	62	CAG GTA GAT ATA ATG CGT CAC CGT GCT TAC ACT CGT TTT ATT AGT ATG TC
8-21	63	CCC TAC AAC ACC ACT GGG CCC AAT TAG ATT AAC GCT ATT TTA TAA CTC G
8-12	64	CCA AAC GGT TAT AAG ACT GAA AAC TCA ATC

```
                              AAT AGC CCA ATC CTC GCC C
        8-13        65        CAC ATG TAT ACC TAA GAA ATT GGT CCC GTA
                              GAC GTC ACA GAC TTA CGC CA
        8-23        66        CAC AAC GAA AAC AAT CTT CCT TGG CAT ACT
  5                           GGG GAG AAA GTC TGT TGT CC
        8-40        67        CAC ACG AAC ATG TCC ATT AAA TGG CAT TCC
                              GTT TTT CGT TCT ACA TAT GC
        8-24        68        CAG AAC GAG GGT CTT GTA AGA CTA CAC CTC
                              CTC AGT GAC AAT AAT CCT G
 10     8-26        69        CAC TAC AGC CTG ATA TAT ATG AAG AAC AGG
                              CAA CAA GCT TAT GCA CTG G
        8-27        70        GGG TAC ATT TAT GAT TCT CTT ATA AAG AGA
                              ATA TCG TAC TCT TTT CCC CA
        8-28        71        CCA AAG TAC ATT CCA ACC CCT TAT ACG TGA
 15                           AAC TTC CAG TAG TTT CCT A
        8-29        72        CTT GAA GAT CCT CAT AAG ACG ATT AAA CAA
                              TCC ACT GGA TAT AAT CCG GA
        8-34        73        CGA ATA GTG TCC ATG ATT ACA CCA ATA ACT
                              GCC TGC CTA TCA TGT TTA TG
 20     8-35        74        CCA AGA GAG TAT CGG ATA CAC TTG GAA CAT
                              AGC TAA CTC GAA CTG TAC CA
        8-36        75        CCA CTG ATA AAT AGG TAA CTG TCT CAT ATC
                              TGC CAA TCA TAT GCC GTA
        8-37        76        CCC AAA TTA TAA ACA ATT TAA CAC AAG CAA
 25                           AAG GAG GTT CAT TGC TCC GC
        8-39        77        CAA TAA ACT GGT GCT AAA CCT AAT ACC TTG
                              TAT CCA AGT TAT CCT CCC CC
```

30   [1]   identical to 10-4, 10-40

         [2]   identical to 8-20, 8-32, 8-38, 10-1, 10-34; 1
mutation to 10-11; 3 mutations to 10-29

35   **Table 3**

Cloned Individuals from 10th Round of Amplification

	Clone No.	SEQ ID NO	"N$_{50}$" Nucleotide Sequence (5'→3')
5	10-3[3]	78	CCG AAT GAC ATC CGT AGT GGA ACC TTG CTT TTG ACA CTA AGA AGC TAC AC
	10-10	79	CCA TAA CAA ATA CCA TAG TAA AGA TCT GCA TTA TAT TAT ATC GGT CCA CC
10	10-12	80	CAG AAC AAA GAT CAG TAG CTA AAC ATA TGG TAC AAA CAT ACC ATC TCG CA
	10-14	81	CCT TTA GTT AGG CTA GCT ACA ACG ATT TTT CCC TGC TTG GCA ACG ACA C
	10-15	82	CTC CCT ACG TTA CAC CAG CGG TAC GAA TTT TCC ACG AGA GGT AAT CCG CA
15	10-19	83	CGG CAC CTC TAG TTA GAC ACT CCG GAA TTT TTC CCC
	10-39	84	CGG CAC CTC TAG TTA GAC ACT CCG GAA TTT TAG CCT ACC ATA GTC CGG T
20	10-23	85	CCC TTT GGT TAG GCT AGC TAC AAC GAT TTT TCC CTG CTT GAA TTG TA
	10-27[4]	86	CCC TTT GGT TAG GCT AGC TAC AAC GAT TTT TCC CTG CTT GAC CTG TTA CGA
	10-31	87	CCT TTA GTT AGG CTA GCT ACA ACG ATT TTT CCC TGC TTG GAA CGA CAC
25	10-18	88	CAT GGC TTA ATC ATC CTC AAT AGA AGA CTA CAA GTC GAA TAT GTC CCC CC
	10-20	89	CAA CAG AGC GAG TAT CAC CCC CTG TCA ATA GTC GTA TGA AAC ATT GGG CC
30	10-6	90	TAC CGA CAA GGG GAA TTA AAA GCT AGC TGG TTA TGC AAC CCT TTT CGC A
	10-7	91	CTC GAA ACA GTG ATA TTC TGA ACA AAC GGG TAC TAC GTG TTC AGC CCC C
	10-8	92	CCA ATA ACG TAA CCC GGT TAG ATA AGC ACT TAG CTA AGA TGT TTA TCC TG

```
        10-16      93       CAA TAC AAT CGG TAC GAA TCC AGA AAC ATA
                            ACG TTG TTT CAG AAT GGT CC
        10-21      94       GCA ACA ACA AGA ACC AAG TTA CAT ACA CGT
                            TCA TCT ATA CTG AAC CCC CA
  5     10-24      95       CCT TTG AGT TCC TAA ATG CCG CAC GGT AAG
                            CTT GGC ACA CTT TGA CTG TA
        10-28      96       CAA AGA TCT CAC TTT GGA AAT GCG AAA TAT
                            GTA TAT TCG CCC TGT CTG C
        10-33      97       CCA CGT AGA ATT ATC TGA TTT ATA ACA TAA
  10                        CGC AGG ATA ACT CTC GCC CA
        10-35      98       CAC AAG AAA GTG TCG TCT CCA GAT ATT TGA
                            GTA CAA GGA ACT ACG CCC
        10-36      99       CAT GAA GAA ATA GGA CAT TCT ACA GGC TGG
                            ACC GTT ACT ATG CCT GTA GG
  15    10-37      100      CAT AGG ATA ATC ATG GCG ATG CTT ATG ACG
                            TGT ACA TCT ATA CCT T
        10-38      101      CAG ATG ATC TTC CTT TAA AGA CTA CCC TTT
                            AAA GAA ACA TAA GGT ACC CC
```

_____

20   ³ 1 mutation to 10-5

        ⁴ 1 mutation to 10-30

        The self-cleavage activity of various clones was
25 subsequently measured. Clones 8-5, 8-17, and 10-3 were (SEQ ID NO 49)
    found to cleave efficiently at the site 5' GUAACU↓AGAGAU 3',
    while clones 10-14, 10-19 and 10-27 were found to cleave (SEQ ID NO 49)
    efficiently at the site 5' G↓UAACUAGAGAU 3'. When the RNA
    portion of the molecule was extended to the sequence 5'
30 GGAAAAAGUAACUAGAGAUGGAAG 3' (residue nos. 1-24 of SEQ ID NO
    51), clones 8-17, 10-14, and 10-27 retained full activity,
    while clones 8-5, 10-3, and 10-19 showed diminished
    activity. Subsequently, clone 10-23 was found to exhibit a
    high level of activity in the self-cleavage reaction

involving the extended RNA domain.

It should also be noted, in the event one of skill in the relevant art does not appreciate same, that the nucleotide sequences preceding and following the "N_{50}"

5   segments of the polynucleotide molecules engineered according to the teachings of the present invention disclosure, i.e, the substrate binding regions flanking the "N_{50}" region, may be altered in a variety of ways in order to generate enzymatic DNA molecules of particular

10   specificities, such as by length, nucleotide sequence, type of nucleic acid, and the like. For example, while residue nos. 1-24 of SEQ ID NO 51 are described herein as RNA nucleotides, they may alternatively comprise DNA, RNA, or composites thereof. (Thus, for example, SEQ ID NO 51 could

15   easily be altered so that nucleic acid residue nos. 1-7 would comprise DNA, residue nos. 8-19 would comprise RNA, residue nos. 20-99 would comprise DNA, and so on.) Similarly, the nucleotides following the "N_{50}" region may comprise RNA, DNA, or composites thereof. The length of the

20   regions preceding and following the "N_{50}" (or "N_{40}" -- see Example 4) region(s) may also be varied, as disclosed herein. Further, sequences preceding and/or following N_{50} or N_{40} regions may be shortened, expanded, or deleted in their entirety.

25   Moreover, as noted above, we selected a specific region of HIV-1 RNA as the target sequence in the methods described in this Example; such a sequence is not the only sequence one may use as a target. Clearly, one of skill in the relevant art may follow our teachings herein to engineer and

30   design enzymatic DNA molecules with specificity for other target sequences. As disclosed herein, such target sequences may be constructed or inserted into larger sequences comprising DNA, RNA, or composites thereof, as

illustrated by SEQ ID NOS 50 and 51.

The self-cleavage reaction was easily converted to an
intermolecular cleavage reaction by dividing the enzyme and
substrate domains into separate molecules. Clones 8-17 and
5   10-23 were chosen as prototype molecules. Both were shown
to act as DNA enzymes in the cleavage of a separate all-RNA
substrate in a reaction that proceeds with multiple turnover
(Figure 8). The substrate binding arms were subsequently
reduced to 7 base-pairs on each side of the unpaired
10   nucleotide that demarcates the cleavage site (Figure 9).

Figure 8 illustrates the nucleotide sequences, cleavage
sites, and turnover rates of two catalytic DNA molecules of
the present invention, clones 8-17 and 10-23. Reaction
conditions were as shown, namely, 10mM Mg^{2+}, pH 7.5, and
15   37°C. The DNAzyme identified as clone 8-17 is illustrated
on the left, with the site of cleavage of the RNA substrate
indicated by the arrow. The substrate sequence (5' -
GGAAAAAGUAACUAGAGAUGGAAG - 3') -- which is separate from the
DNAzyme (i.e., intermolecular cleavage is shown) -- is
20   labeled as such. Similarly, the DNAzyme identified herein
as 10-23 is shown on the right, with the site of cleavage of
the RNA substrate indicated by the arrow. Again, the
substrate sequence is indicated. For the 8-17 enzyme, the
turnover rate was approximately 0.6 hr^{-1}; for the 10-23
25   enzyme, the turnover rate was approximately 1 hr^{-1}.

As illustrated in Figure 8, the nucleotide sequence of
the clone 8-17 catalytic DNA molecule capable of cleaving a
separate substrate molecule was as follows:
5'-CTTCCACCTTCCGAGCCGGACGAAGTTACTTTTT-3' (residue nos. 1-34
30   of SEQ ID NO 56). In that same figure, the nucleotide
sequence of the clone 10-23 catalytic DNA molecule capable
of cleaving a separate substrate molecule was as follows:
5'-CTTTGGTTAGGCTAGCTACAACGATTTTTCC-3' (residue nos. 3-33 of

SEQ ID NO 85).

Figure 9 further illustrates the nucleotide sequences, cleavage sites, and turnover rates of two catalytic DNA molecules of the present invention, clones 8-17 and 10-23. Reaction conditions were as shown, namely, 10mM Mg^{2+}, pH 7.5, and 37°C. As in Figure 8, the DNAzyme identified as clone 8-17 is illustrated on the left, with the site of cleavage of the RNA substrate indicated by the arrow. The substrate sequence (5' - GGAAAAAGUAACUAGAGAUGGAAG - 3' which is separate from the DNAzyme (i.e., intermolecular cleavage is shown) -- is labeled as such. Similarly, the DNAzyme identified herein as 10-23 is shown on the right, with the site of cleavage of the RNA substrate indicated by the arrow. Again, the substrate sequence is indicated. For the 8-17 enzyme, k_{obs} was approximately 0.002 min^{-1}; for the 10-23 enzyme, the value of k_{obs} was approximately 0.01 min^{-1}.

As illustrated in Figure 9, the nucleotide sequence of the clone 8-17 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows: 5'-CCACCTTCCGAGCCGGACGAAGTTACT-3' (residue nos. 4-30 of SEQ ID NO 56). In that same figure, the nucleotide sequence of the clone 10-23 catalytic DNA molecule capable of cleaving a separate substrate molecule was as follows: 5'-CTAGTTAGGCTAGCTACAACGATTTTTCC-3' (residue nos. 5-33 of SEQ ID NO 85, with "CTA" substituted for "TTG" at the 5' end).

The catalytic rate of the RNA-cleaving DNA enzymes has yet to be fully optimized. As disclosed above and as reported in previous studies, we have been able to improve the catalytic rate by partially randomizing the prototype molecule and carrying out additional rounds of selective amplification. We have found, however, that the K_m for Mg^{2+} is approximately 5 mM and 2 mM for the 8-17 and 10-23 DNA

enzymes, respectively, measured at pH 7.5 and 37°C; this is certainly compatible with intracellular conditions.

6.   Preparation of of a Universal Substrate Enzyme

5   The foregoing has shown that an enzyme of the present invention has the ability to catalytically cleave target nucleic acids having preselected sequences. Furthermore, an enzyme is shown to cleave a nucleic acid which is all DNA, or which has a ribonucleotide (i.e., an RNA component)

10   imbedded within a deoxyribonucleotide (i.e., DNA) sequence, or which is entirely DNA. In addition, it is seen that the substrate can be altered and an enzyme is prepared which can cleave that substrate.

         By the present invention, an enzyme is provided that

15   can cleave any targeted substrate, i.e., one containing a preselected nucleotide sequence in the length range of about 10-30 nucleotides, in a kinetic manner that is efficient and specific under physiological conditions. This enzyme is easy to manufacture and inexpensive, and is useful for

20   inactivation of target RNA, particularly messenger RNA (mRNA), for probing the structure of RNA and for the manipulation of recombinant RNA, such as a sequence-specific endoribonuclease.

         For inactivation of RNAs, the enzyme is particularly

25   useful for inactivation of target cellular RNAs, such as an "antisense" oligodeoxynucleotide can be used, to block mRNA function. However, the present enzyme is superior to an antisense reagent because the present enzyme provides both target recognition and cleavage, and can function with

30   catalytic turnover, whereas an antisense molecule only provides recognition in a equimolar manner (i.e. non-catalytic), and must rely upon other cellular enzymes for the RNA inactivation reactions.

-87-

The following section describes the preparation of improved enzymes based on the "10-23" and the "8-17" motifs described above. These improved enzymes are generic enzymes which can cleave any preselected target sequence, and that

5 target specificity depends solely on the sequence of the substrate binding regions of the enzyme, as described further herein.

As described in Example 5, above, two motifs, designate "10-23" and "8-17", were identified during sequential rounds

10 of selective amplification and recast into an intermolecular cleavage reaction, and shown to perform efficiently in this manner.

Further studies were conducted using a reaction that yielded site-specific catalytic cleavage of the separate

15 substrate molecule shown in Figure 9 (i.e, intermolecular cleavage) under simulated physiological conditions of 2 mM MgC12, 150 mM KCl, pH 7.5, 37 C, for a rate of about k_{cat} = 0.01 min^{-1} .

Cleavage occurred following an unpaired purine

20 nucleotide of the substrate that was flanked by oligonucleotides complementary to the enzyme. The 5' and 3' cleavage products bore a 2'(3') phosphate and 5' hydroxyl, respectively, indicative of a reaction mechanism involving attack by a 2⊣ hydroxyl on an adjacent phosphate. For both

25 the 8-17 and 10-23 motif enzymes, the sequence of the substrate can be changed without loss of catalytic activity, so long as the substrate-binding arms of the enzyme were changed in a complementary manner. The 8-17 enzyme had a special requirement for a rG-dT "wobble" pair located

30 immediately downstream from the cleavage site. Substitution of a Watson-Crick pair at this position eliminated catalytic activity. The substrate-binding arms of the 10-23 enzyme interacted with the substrate entirely through standard

Watson-Crick pairing. The catalytic core of the 8-17 and
10-23 motif enzymes, located between the two
substrate-binding arms, contained 13 and 15
deoxynucleotides, respectively.

5   In order to define more precisely the sequence
requirements of the catalytic core, a library of 10^{14}
variants of each motif was generated, introducing random
mutations at a frequency of 25% per nucleotide position
throughout the core. Each library was subjected to six

10   different in vitro selection protocols, involving a total of
52 rounds of selective amplification. The method and
stringency of selection were varied in order to conduct a
thorough examination of sequences related to the two
prototype molecules. Individuals from the selected

15   populations were cloned, sequenced, and tested for catalytic
activity. This procedure was carried out as follows.

The re-selections based on the 8-17 and 10-23 molecules
involved six different lineages for each motif. Each lineage
entailed 5-21 rounds of in vitro selection, differing with

20   respect to the selection protocol and reaction times. All
cleavage reactions were carried out in 2 mM MgCl2, 150 mM
NaCl, and 50 mM Tris*HCl (pH 7.5) at 37 C. Reaction times
varied from 60 min in early rounds to 1 min in later rounds.
Each starting pool of templates was based on a sequence

25   complementary to the prototype, with fixed binding arms of
seven nucleotides each and a catalytic core randomized to
25% degeneracy at each nucleotide position. For the 8-17 and
10-23 motifs, the templates had the
sequence

30   5'-gtgccaagcttaccgagtaactTCG-TCCGGCTCGGRagatgggtcgtctgtccttc
cATCTCTAGTTACTTTTTC- 3' and (SEQ ID NO 124)

5'-gttgccaagcttaccg-ggaaaaaTCGTTGTAGCTAGCCtaactaggtcgtctgtcc
ttccATCTCTAGT TACTTTTTC-3', respectively (PCR primer sites
(SEQ ID NO 125)

in lower case; substrate-binding arms underlined; randomized
positions italicized). The primer used in the
template-directed extensions had the sequence
5'-biotin-r(GGAAAAA-GUAACUAGAGAUGG)d(AAGAGATGGCGAC)-3'. The
PCR primers for the 8-17-based selections were
5'-GTGCCAAGCTTACCGAGTAACT-3' and
A^{14} (SEQ ID NO 127) insert
5'-d(GGAAGGACAGACGACC-CATC)rU and for the 10-23-based
(SEQ ID NO 128)
selections were 5'-GTGCCAAGCTTACCGGGAAAAA-3' and
5'-d(GGAAGGACAGACGACCTAGTT)rA. The PCR primers encompassed
the binding arms, thus fixing these sequences. One of the
PCR primers in each set contained a 3'-terminal
ribonucleotide, allowing isolation of the template strand
from the double-stranded PCR products by alkaline hydrolysis
of the non-template strand and subsequent purification by
polyacrylamide gel electrophoresis. A gel-based selection
scheme was employed in some of the lineages. In those A^{15}
cases, the PCR primers were 5'-biotin-GTGCCAAGCTTACCG-3' and
(SEQ ID NO 129) insert
5'-GAAAAAGTAACTAG-AGATGGAAGGACAGACGACC-3' and the extension
reactions were carried out on the solid support using the
A^{16}
primer 5'-r(GGAAAAAGUAACUAGAGAUGGAAG)-3'. A trace amount of
insert
[a-32P]-dATP was included in the mixture to label the
extension products, which were eluted with alkali, purified
by denaturing polyacrylamide gel purification, and recovered
by electroelution. The molecules then were reacted and
those that underwent cleavage were isolated by gel
electrophoresis.

    Following the eighth and tenth rounds of the initial
selection, individual molecules were cloned and sequenced,
as described above. The 17th clone from round eight (8-17)
and 23rd clone from round ten (10-23) had the sequence
5'-cacggttcga-atggcGTTATGCATCACACTATTTTTCATTGAAGCAGGCCGAGCCT
(SEQ ID NO 130)
T CCACCTTCcagcggtag-agaagg-3' and
5'-cacggttcgaatggcATGTTAAGTTCGTCCCTTTTTAGCAACATCGATCGGATT-GG

(SEQ ID NO 131)

TTTCCCcagcggtagagaagg-3', respectively (primer sites in
lower case; substrate-binding arms underlined). For the
intermolecular reaction, synthetic oligodeoxynucleotides
were prepared based on the cloned sequences but lacking the

5   regions outside of the substrate-binding arms. These were
used to cleave an all-RNA substrate having the same sequence
as the primer used to construct the initial library (see
above). Subsequently, the substrate-binding arms of the DNA
enzyme were reduced to seven nucleotides each and made

10   perfectly complementary to the RNA substrate.

        In the case of the 8-17 enzyme, sequence variation
among the cloned individuals suggested that the catalytic
core consisted of a short internal stem-loop followed by an
unpaired region of 4-5 nucleotides (Figure 10). The stem

15   always contained three base-pairs, at least two of which
were G-C. The loop was invariant, having the sequence
5'-AGC-3'. Synthetic constructs in which the stem was
lengthened or the sequence of the loop was altered did not
exhibit catalytic activity. The unpaired region, connecting

20   the 3' half of the stem to the downstream substrate-binding
domain, had the sequence 5'-WCGR-3' or 5'-WCGAA-3' (W = A or
T; R = A or G). Variants having the sequence 5'-TCGAA-3' in
this region exhibited the highest level of catalytic
activity, but this enhancement relative to the 8-17 enzyme

25   was not generalizable to other substrate sequences.

        The eighth nucleotide position of the catalytic core of
the 10-23 enzyme motif allows variation as either T, C, or
A, although a T at this position (as in the prototype)
provided the highest level of activity. A survey of numerous

30   different combinations of RNA substrate and corresponding
complementary DNA enzyme in the substrate binding region
revealed that the 10-23 motif was generalizable with respect
to any substrate sequence.

Catalytic activities for the 10-23 and 8-17 motif
variants were then measured using multiple-turnover
reactions, typically exhibiting < 20% variation for
identical experiments performed on different days. Kinetic
5   values obtained in single- and multiple-turnover experiments
were similar; values obtained with synthetic RNA substrates
were slightly less favorable than those obtained with in
vitro transcribed substrates. Reported kcat and Km values
were determined from the y-intercept and negative slope,
10   respectively, of the best-fit line to a modified
Eadie-Hofstee plot of kobs vs. kobs / [S]. Each plot
consisted of ten data points for a range of [S] that spanned
Km, with [S] in ä 10-fold excess over [E]. kobs values were
typically based on five data points obtained over the first
15   10% of the reaction. Substrate and enzyme molecules were
preincubated separately for 10 min in reaction buffer, then
combined to initiate the reaction. All reactions were
carried out in the presence of 0.01% sodium dodecyl sulfate
to prevent adherence of material to the vessel walls. The pH
20   was maintained by addition of 50 mM
4-(2-hydroxyethyl)-piperazine-1-propanesulfonic acid.
Kinetic values did not depend on the identity of the buffer.
Reaction products were separated by electrophoresis in a
denaturing 20% polyacrylamide gel and quantitated
25   using a phosphorimager.

Cleavage occurred on the 3' side of a single unpaired
nucleotide, preferably a purine, that was followed by a
pyrimidine. Target sites surrounded by A and U were cleaved
most efficiently, with a catalytic rate of approximately 0.1
30   min-1 under simulated physiological conditions.

A DNA enzyme that cleaves RNA at an A*U site can be
used to target any mRNA start codon (A*UG). As a test case,
both synthetic and in vitro transcribed versions of a 17mer

RNA corresponding to the translation initiation region of
HIV-1 gag/ pol mRNA (5'-GGAGAGAGA*UGGGUGCG-3') were
(SEQ ID NO 102)
prepared. Both versions of the substrate were cleaved at
the expected position by the corresponding 10-23 DNA enzyme,
in a reaction that proceeded with a kcat of 0.15 min-1 and
Km of 0.47 nM under simulated physiological conditions
(catalytic efficiency, kcat / Km = 3.2 x 108 M-1 min-1)
(Figure 11). The catalytic rate increased with increasing
MgCl2 concentration range of 1-250 mM, with an apparent Km
for Mg2+ of 180 mM at pH 7.5 and 37 C. The catalytic rate
increased in a roughly log-linear fashion with increasing pH
over the range 7.0-8.5 consistent with a reaction mechanism
involving deprotonation of the 2'-hydroxyl that lies
adjacent to the cleaved phosphoester. In the presence of 50
mM MgCl2 at pH 8.0 and 37 C, conditions that are useful in
the laboratory manipulation of RNA, kcat was 3.4 min-1 and
Km was 0.76 nM. The catalytic efficiency of the 10-23 DNA
enzyme, under both physiological and laboratory conditions,
compares favorably with that of known RNA-cleaving RNA
enzymes. Compared to the protein enzyme ribonuclease A, the
DNA enzyme has ~104-fold lower kcat but ~105-fold more
favorable Km.

The 10-23 enzyme can be used to cleave a variety of
biologically relevant RNAs. We prepared synthetic RNA
substrates corresponding to 15-17 nucleotides surrounding
the translation initiation site of HIV-1 gag / pol, env,
vpr, tat, nef IGF-R and E100 ligase mRNA. Each was cleaved
at the expected position by a synthetic DNA enzyme that
contained the 10-23 catalytic core flanked by
substrate-binding arms of 7 to 12 nucleotides each (Table
4). In all cases the catalytic rate was about 0.1 min-1
under simulated physiological conditions. The value for Km,
however, varied with the nucleotide composition of the

substrate. For the guanosine-rich gag / pol substrate, Km
was < 1 nM when either the 7- or 8-nucleotide
substrate-binding arms were employed. The env and vpr
substrates were cleaved with a much less favorable Km when
5   the 7-nucleotide binding arms were used, but Km improved
substantially when the arms were increased to 8 nucleotides
each.

TABLE 4

10   DNA-catalyzed cleavage of various RNA substrates under
simulated physiological conditions

Gene	SEQ ID NO	Target Sequence	Arm length	kcat (min-1)	Km (nM)
HIV-1					
gag	102	GGAGAGAGA*UGGGUGCG	8+8	0.1	0.7
gag	103	GAGAGAGA*UGGGUGC	7+7	0.1	0.9
env	104	CAGUGGCAA*UGAGAGUG	8+8	0.04	9
env	105	AGUGGCAA*UGAGAGU	7+7	0.03	900
vpr	106	GAGGAUAGA*UGGAACAA	8+8	0.1	20
vpr	107	AGGAUAGA*UGGAACA	7+7	0.08	500
tat	108	GCAAGAAA*UGGAGCC	7+7	0.04	300
nef	109	CUAUAAGA*UGGGUGA	7+7	0.05	900
FIV					
gag	110	UACAGCAACA*UGGGGAAUGG	9+10	0.005	8
gag	111	CAUGGGGAA*UGGACAGGG	8+9	0.006	5
IGF-R					
	112	CAAAUAAAAGGGA*UGAAGUCUGG	12+10	0.02	20
	113	AAGGAAUGAAG*UCUGGCUCCG	10+10	0.3	50
	114	AUACCGCAAAG*UCUUUGAGAAUU	10+12	0.1	30
	115	AAGUCUUUGAGAG*UUUCCUGCAC	12+10	0.05	21
	116	AACACCACCA*UGUCCAGCC	9+9	0.06	2

117 GGCCUUUCACA*UUGUACCGC   10+9 0.1   10

118 UUGUACCGCA*UCGAUAUCCAC   9+11 0.06 1

E100 ligase

119 GAACAUUACAUUA*UAGUGACCAG 12+10 1.0   80

Kinetic values measured and shown in Table 4 were
obtained under multiple-turnover conditions, with synthetic
RNA substrate in >10-fold excess over synthetic DNA enzyme,
using the reaction conditions: 2 mM MgCl2, 150 mM NaCl, pH
7.5, 37 C, obtained at 25 mM MgCl2.

As a further demonstration of the variation to the
substrate binding region (arms) that a DNA enzyme of the
present invention can support, the length of the arms was
systematically varied from 4 to 13 nucleotide residues using
the HIV-1 gag gene start codon target nucleotide sequence
shown in Table 4. The reaction was run separately for each
DNA enzyme construct prepared as described above except that
the arm length was varied as shown in Figure 13. The
kinetic properties of the catalytic DNA enzyme was measured
as before under the conditions of 2 mM Mg+2, 150 mM NaCl, pH
7.5, 37 C and measuring multiple turnover of enzyme. Both
k_{cat} (min^{-1}) and K_m (nM) was measured, and is shown in Figure
13 using the 10-23 motif modified for complementary binding
to the HIV-1 gag gene.

The results show that useful catalytic rates are
observed over the full range of from 5 to 13 nucleotides per
arm per substrate binding region, with a preferred range of
from 7 to 10 nucleotides being particularly efficient. The
results also show that the effective concentration of enzyme
for half maximal catalysis (K_m) is in the range of 1000 to
0.05 nanomolar (nM) for arms of a 5 to 13 nucleotide length,
with lengths of 7 to 13 particularly preferred.

-95-

A variety of modifications were incorporated into the
10-23 DNA enzyme and tested for stability in 10% fetal
bovine serum (Figure 13). These modifications included: 1)
an "inverted" (3',3'-linked) thymidylate at the 3' end of
5   the DNA; 2) five 2'-O-methyl residues at the distal end of
both substrate-binding arms; 3) 2'-O-methyl residues at all
positions in both substrate-binding arms; 4)phosphorothioate
residues at the five pyrimidine-pyrimidine sites within the
catalytic core; 5) three phosphorothioate residues at the
10   distal end of both substrate-binding arms. The best
protection was afforded by the inverted thymidylate (t1/2 >
60 min). All of the other modifications resulted in enhanced
serum stability compared to the unmodified DNA, with the
exception of the five P=S substitutions in the core. All of
15   the modified DNA enzymes retained catalytic activity.

Thus, the invention describes a catalytic DNA molecule
having site-specific endonuclease activity which can be
designed to cleave any preselected substrate nucleic acid
20   sequence. The DNA molecule (enzyme) has first and second
substrate binding regions (arms) flanking a core region, and
each arm has a sequence complementary to a portion of the
target substrate nucleic acid sequence, such that together,
the first and second arms define the substrate nucleic acid
25   sequence to be cleaved. By complementary is meant that the
substrate binding regions bind to the target sequence using
conventional Watson-Crick base pairing.
The arms can be any of a variety of lengths. However,
because it is seen that the length affects the catalytic
30   rate (k_{cat}) and effective concentration (K_m) of the enzyme
(see Figure 12), the preferred arm lengths are from 5 to 13
nucleotides of complementarity each, preferably about 6 to
11 nucleotides, and more preferably about 7 to 10

nucleotides in length.

The core region can have a variety of sequences based
on the formula:

(I.)   T(stem)'AGC(stem)"Z,

wherein said (stem)' and (stem)" are each three sequential
nucleotides which when hybridized as a (stem)':(stem)" pair
comprise three base pairs including at least two G:C pairs
and wherein said Z = WCGR or WCGAA, and W = A or T and R = A
or G. This stem structure is shown in Figure 10 and
illustrates three complementary nucleotide pairs.
Particularly preferred is the prototype structure of the
core region of motif 8-17, "TCCGAGCCGGACGA" (SEQ ID NO 120)
shown in Figure 10.

In another embodiment, the core region can have a
sequence according to the formula:
$RGGCTAGCHACAACGA$
(II.)   R̶G̶G̶C̶T̶A̶G̶C̶X̶A̶C̶A̶A̶C̶G̶A̶ (SEQ ID NO 122),
wherein said X = T, C or A, and R = A or G. Particularly
preferred is the prototype structure of the core region of
motif 10-23, "RGGCTAGCTACAACGA" (SEQ ID NO 121) shown in
Figure 10.

A DNA enzyme of the above design can exhibit a range of
useful catalytic rates and effective catalytic
concentrations depending upon the arm length as noted in
Figure 12. A DNA enzyme of this invention according to the
8-17 or 10-23 motif typically has a catalytic turnover rate
(k_{cat}) of about 0.005 to 0.1 min^{-1}, preferably about 0.01 to
0.1 min^{-1}, and more preferably about 0.03 to 0.1 min^{-1}, under
physiological conditions. A particularly preferred enzyme
has a rate of about 0.1 min^{-1}. A DNA enzyme of this
invention according to the 8-17 or 10-23 motif typically
also has a catalytic half maximal effective concentration
(K_m) of about 0.05 to 1000 nanomolar (nM), preferably about
0.7 to 900 nM, more preferably less than about 1.0

micromolar (uM), and most preferably about 0.1 (uM), under
physiological conditions.

      The foregoing also demonstrates that a preferred DNA
enzyme has nucleotide modifications which stabilize the DNA

5   enzyme for use under physiological conditions. Any of a
variety of modifications can be utilized so long as the
Enzyme retains its catalytic activity as defined and
measured herein, and therefore the invention need not be so
limited to a particular stabilitation modification.

10   Preferred modifications render a subject DNA molecule less
susceptible to exo or endo nucleolytic digestion. In one
embodiment the modification comprises incorporation of one
or more nucleoside phosphorothioate residues, such as is
described by Zhang et al, Biochem. Pharmacol., 50:545-556

15   (1995) or by Stein, C.A., Trends Biotechnol., 14:147-149
(1996). The phosphorothioate residue can be in the arms or
in the core, and in one embodiment can comprise a residue on
a dipyrimidine within the core. Another modification
comprises the substitution of an O-methyl group onto the 2'

20   position of the ribose or deoxyribose component of the sugar
nucleotide, to form a 2' O-methyl ribonucleotide, as well
known and described by Zhang et al., above. An additional
modification is to attach an inverted terminal nucleotide
onto the 3' end of the DNA molecule, such that the 3' end is

25   blocked, appearing as a free 5' end. This structure blocks
3'endonucleases, and is produced by forming a 3'-3' linked
nucleotide at the 3' end (i.e, an inverted nucleotide). The
preparation of a 3' inverted nucleotide at the 3' end is
well known, and is prepared using a modified solid support

30   in oligonucleotide synthesis having the terminal 3' residue
inverted as a starting material. A preferred solid support
material for this purpose is dT-5'-CPG 500, available from
Glen Research (Sterling, VA) and is a modified controlled

pore glass resin having the modified residue.

The foregoing specification, including the specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.